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NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

ANNUAL REPORT

July 1, 1975 through June 30, 1976

PART II

PART II

INTRAMURAL RESEARCH PROGRAMS

United States, NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

ANNUAL REPORT of program activities
July 1, 1975 through June 30, 1976

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NICHD Annual Report

July 1, 1975 through June 30, 1976
Office of the Scientific Director
Intramural Research Programs

The Intramural Research Program continues to be productive. Many of the projects are exceedingly imaginative and sophisticated, being on the forefront of the specific area of science.

The constraints of space, positions, and dollars on research in the National Institute of Child Health and Human Development in the Intramural Program have been felt even more keenly this past year than the previous years. This has been particularly keen in that there has been an attempt to establish new initiatives in the area of pediatrics and obstetrics which has required additional resources. Our position ceiling was set at 187, and this has required very stringent administrative restraints to reduce the numbers to come within the allotted ceiling. In addition to the full-time positions, we have approximately 71 scientists working in NICHD laboratories as guest workers (36) and visiting fellows (35). There are approximately 55 employees in the category of temporary, part-time, WAE, stay-in-school, junior fellow, or work-study categories.

Intramural budget for FY 1976 was $8,540,000, up $640,000 from FY 1975. The FY 1976 and FY 1975 amounts do not include the management fund contribution which is estimated for FY 76 at $4,600,000. This amount was apportioned as $5,440,000 for salaries and $3,100,000 for other objects support. The other objects monies were obligated as follows: laboratory renovation $140,000, equipment, $773,000, travel $90,000, data processing $150,000, and $2,547,000 for all other operating expenses.

The primary organizational change which was effected in the current year was the separating of the Developmental Pharmacology Branch and creating the Neonatal and Pediatric Medicine Branch under Dr. Philip Farrell with Dr. Joseph Schulman, Chief of the Section on Human Biochemical and Developmental Genetics. In line with the creation of this new branch, we opened Ward 10D in the Clinical Center which is designed and equipped for the care of small infants. This ward was opened on November 26, 1975. Ward 9D, which has been designated for children up to the age of 14, was opened on May 10, 1976. These wards are supervised by board certified pediatricians. The plan is that these wards will serve as a pediatric resource for all investigators in the Clinical Center, both within and outside the NICHD. It is our hope and intent that we will provide high quality pediatric care for the Clinical Center.

During the year, we experienced the loss of a very keen individual in the NICHD in the person of Dr. Griff Ross who has been Chief of the Reproduction Research Branch since the departure of Dr. Mortimer Lipsett; and he also served as Acting Scientific Director for a portion of the past year. Dr. Ross has been recognized for his contributions and unique abilities in being appointed as Acting Director of the Clinical Center. We feel that though this is a severe loss for us, Dr. Ross will give a noble accounting of himself in his new role which will benefit all of the NIH.

Dr. James Sidbury was appointed Scientific Director on August 4, 1975.

During the past year, prior to the opening of Ward 9D, the ward was used as a reservoir to permit the continued operations of other wards in the Clinical Center during a period of renovation and painting of those wards. With the opening of 9D for pediatric service, this now completes the inpatient pediatric inpatient service as such. Further building was underway during the year. The Building 6 Annex is nearing completion and will be expected to be occupied in early FY 77.

The long, anticipated building of Building 33 has been shelved for the present time. The building of the Ambulatory Care Research Facility as as an extension of the Clinical Center is the priority for construction for the NIH. This Ambulatory Care Unit will benefit all institutes. It is still hoped that the NICHD, in time, will see the construction of Building 33, and that all its multi-located programs will be brought together in one area. This would facilitate not only improved facilities, which are less than ideal at the present time, but of equal importance is having the operations in a single area which would permit the development of a much sounder training operation.

Reproduction Research Branch - Accomplishments

The scope of the investigations conducted by these scientists ranges from analysis of physical, chemical, and antigenic properties of purified gonadotropic hormones through studies of the physical and biochemical correlates of hormonal interactions with subcellular organelles and living cells isolated from ovary, testis, and adrenal, to studies of the complex interactions between hypothalamus, pituitary, gonads, and genitalia in the intact animal. The hormonal action is initiated by the binding of hormone molecules to receptors, some in cell membranes and some in cell sap. This receptor-hormone interaction initiates the sequence whereby chemical events which culminate in the characteristic "activities" of hormones upon the gonadal and other tissues which respond to these substances. Much of the research must be directed towards developing new methods for studying the physical and biochemical basis of interactions between hormones and receptors.

Partial digestion of human growth hormone has been shown to increase plasma half life of the modified growth hormone in human subjects, possibly accounting for its enhanced biological activity. Purified prolactin has been isolated from amniotic fluid. Isozymes of the peptide hormones have been demonstrated with the newer techniques for protein and peptide separation.

Nuclear magnetic resonance has been used to demonstrate its validity in structural identifications not otherwise readily approachable. For example, the coliform polysaccharide was shown to represent a hybrid of alpha 2,8 and alpha 2,9-linked heteropolymers of sialic acid, whereas meningococcus group C polysaccharide consists of a pure alpha 2,9-linked polymer sialic acid. In addition, ^{13}C NMR was used to study the conformational surfaces of thiotepa, an agent useful in the treatment of human ovarian cancers. To increase the sensitivity of the NMR method, ^{13}C amino acids are being used to synthesize peptides with known biological activities for further studies. This technology can be applied to studies such as the conformation of the carboxy terminal peptide in human chorionic gonadotropin and the recently demonstrated similar hormone in pituitary extracts.

Studies of human chorionic gonadotropin carried out in the present year are potentially of major importance. The amino acid sequence has been determined for the peptides isolated following tryptic digestion of the S-carboxy methylated desialated beta subunit of human chorionic gonadotropin. This peptide contains 23 carboxy terminal amino acid residues of this hormonal subunit, and this sequence is not duplicated in any other human glycoprotein hormone (hLH, hTSH, or hFSH). Antibody to this peptide is being developed in rabbits. To test the specificity of the antisera, synthetic peptides, containing varying numbers of amino acids in the sequence proposed by Canfield for this tryptic peptide, were used to inhibit the binding of ^{125}I-labeled hCG to these antisera to radioimmunoassays. It has been shown that the last 18 amino acid residues of this peptide are an integral to the antigenic recognition.

During the year, a protein with the properties of the receptor for testosterone has been demonstrated in cell sap from glomerulosa cells derived from preantral follicles of estrogen-primed hypophysectomized immature female rats. In addition, it has been shown that testosterone is translocated to the nuclei of the cells where the steroid is bound to a macromolecule. It is thought that these observations are consistent with the role of androgens in the regulating follicular and atresia in mammalian ovaries. The cell sap from adrenal cortical cells of the guinea pig has been shown to contain a receptor protein for estrogen, providing a basis for the participation of this sex hormone in the control of adrenal cortical androgen synthesis, increases which are concommitant with pubescence in guinea pigs and humans. Two important drugs which cause gynecomastia as a side-effect were shown to interact with cytosol receptors in breast tissue.

Radioligand-receptor assays employing gonadal particulate fractions have been used to compare the biological activity of labeled and unlabeled gonadotropin, making it possible to calculate the specific activity of labeled peptide hormone. This development will contribute to increased accuracy in calculating numbers of affinities and receptor sites during changes such as growth and development. The solubilization and purification of gonadotropin receptors of testis and ovary have been extended to isolation of LH/hCG receptors by affinity chromatography, in a state of protein theoretical purity. Similar procedures have been applied to solubilization of FSH receptors from the testis tubules. The relationships between solubilized LH receptors and adenylate cyclase were studied in detergent extracts of testis and ovary, and suggest that a physical association between receptors and cyclase may exist in solution. Suspensions of intact Leydig cells from rat testes have been shown to respond to physiologic concentrations of LH and hCG by secretion of testosterone into the incubation medium. This action of gonadotropins on steroid hormone secretion has been found to require continuous RNA and peptide synthesis by these cells. Studies in cell suspensions suggest that estrogen modulates the response of the corpus luteum to gonadotropins. Electron microscope studies in eutheria, as in vertebrates, the second cleavage plane is perpendicular to the first, resolving an apparent paradoxical discrepancy of these two orders.

Studies in pregnant animals have shown that the placenta secretes an adrenocorticotrophic substance which maintains adrenal weight and prevents decline in maternal serum cortisol levels following maternal hypophysectomy. The maternal hypophysis appears to contribute to the control of serum estradiol levels in both fetal and maternal compartments during gestation in the monkey.

Reagents developed for detecting and measuring chorionic gonadotropin in rhesus monkey serum have been applied to detection of the hormone in these fluids from other primates. To date, these reagents have been found useful in diagnosing pregnancy in baboons, marmosets, and chimpanzees in addition to their use in reproduction research in the rhesus. More than 400 kits containing these reagents for 40 tests each have been distributed to reproductive biologists around the world. False-positive and false-negative tests are less than 5% in extensive field trials reported to date.

A very sensitive in vitro bioassay for LH/hCG in specimens of human biological fluids has been developed. It has been found that the ratio of immune potency to biological potency is unity in normal women throughout the menstrual cycle, whereas in men and postmenopausal women the ratios are 2 or 3 to 1. The basis for this discrepancy will be investigated.

Studies of angiotensin II receptors and the control of aldosterone synthesis in zona glomerulosa cells bearing the properties of angiotensin II receptors in dog and rat adrenal homogenates and cell suspensions were defined, and occupancy of receptors by angiotensin II were shown to be correlated with glomerulosa cell activation and aldosterone production in vitro. The relationship to sodium balance on these parameters was demonstrated.

In the clinical area, studies continue regarding the condition, anorexia nervosa in young women, and have led to the conclusion that the disease has a large component of disordered hypothalamic function. A drug cyproheptadine, has been shown to be effective in a number of these young women.

Neonatal and Pediatric Medicine Branch - Accomplishments

1. Study of the process of lung maturation - The regulatory mechanisms involved in the maturation of developing lung have been studied with particular reference to surface-active choline phosphoglycerides. Various hormones (cortisol and thyroxin) have been studied from the view of determining the effect on the rate of maturation and production of surfactants.

2. Unit on Developmental Endocrinology and Metabolism: Mineral nutrition and hypocalcemia have been problems of interest in the premature and term newborn infant, and a method has been developed for measuring the metabolism of calcium utilizing stable calcium isotopes in the small infant.

3. The major effort in terms of time of this branch has been spent in opening Wards 9 and 10 during the year and working out the problems related to developing full efficiency of the wards.

4. Section on Human Biochemical and Developmental Genetics: Two patients have been discovered with inborn errors of metabolism characterized by an inability to break down glutathione, i.e., gamma-glutamyl transpeptidase deficiency. Using cells from these patients, it has been possible to test the hypothesis if this enzyme is involved in the mammalian amino acid transport. Results indicate that the absence of such enzyme activity, intracellular amino acid transport kinetics and pool sizes remain normal. It has been demonstrated that children with oxyprolinuria, associated with acidosis and marked reduction of glutathione synthase activity of red cells, have genetic alterations in the synthase enzyme. In contrast, patients with the adult form of glutathione synthase deficiency do not have acidosis and can be shown to have an unstable synthase resulting in selective expression of their metabolic defect in the red cells. Studies indicate that work on glutathione synthase in normal and mutant tissues and cells have indicated that different forms of the enzyme exist in red cells, fibroblasts, and kidney. The control clinical trial using ascorbic acid to treat patients with cystinosis continues.

Developmental Pharmacology Branch - Accomplishments

1. The group continues to work on (1) delineation of further differences between the monooxygenase induction by phenobarbital and that by polycyclic hydrocarbons; (2) characterize specific and nonspecific binding of these various inducers to subcellular sites; (3) examine the effects of hormones and nutrition on monooxygenase induction and associated intermediary molecules of importance such as cyclic-AMP, cyclic-GMP, and polyamines; (4) study various inhibitors and activators of monooxygenase activity in cell culture; (5) determine differences in metabolite formation, binding of reactive metabolic intermediates to DNA, and biophysical characteristics of enzyme active-site between control and drug-treated animals or cells in culture; (6) compare these results in genetic differences with contemporary models of mammalian gene expression and regulation; and (7) search for drug metabolism parameters in cultured human lymphocytes or fibroblasts or in various excreta that might aid in predicting a patient's response to a drug or class of drugs.

2. Explore further genetic differences in response to aromatic hydrocarbon treatment between certain inbred strains of mice to determine substrate preferences for the induced transferase; (2) examine urine and bile for glucuronides of labeled exogenous substrates administered to treated responsive and nonresponsive mice; (3) determine the time course for glucuronide and other metabolites of the monooxygenase system, with a comparison of various carcinogenic and noncarcinogenic polycyclic hydrocarbons in the two

genetic strains of mice; (4) characterize further the transferase system in cell culture with respect to macromolecular syntheses requirements, potential inhibitors, and activators and general regulation at the molecular level; (5) determine bilirubin tolerance in responsive and nonresponsive mouse after hydrocarbon treatment; (6) examine the effect of UDP glucuronyl transferase on bacterial mutagenesis; and (7) develop a useful bioassay in cell culture for the quantitation of "breast milk jaundice factor" in human breast milk samples from outpatients and/or inpatients.

3. To delineate further the relationship between aryl hydrocarbon hydroxylase activity and N-hydroxylation of N-acetylarylamines in the inbred strains of mice following pretreatment with polycyclic hydrocarbons; examine in detail the catalytic enzyme activity-site responsible for N-hydroxylation of N-acetylarylamines; explore the possibility of "phenotyping" for aryl hydrocarbon hydroxylase and N-hydroxylase activity in man by measuring the amount of mercapturic acid conjugation following oral administration of acetaminophen; determine the role of the liver microsomal mixed-function amine oxidase in toxicity and mutagenicity; delineate further the role of ovarian monooxygenase in chemically induced ovarian cancer; modify carcinogenicity of N-acetylarylamines by steroids; and determine and examine the effects of vitamin A on DNA repair enzymes in both bacterial and mammalian cells.

Pregnancy Research Branch - Accomplishments

The Pregnancy Research Branch continues to be successful in utilizing the animal model system using in particular the pregnant rhesus monkey, baboon, and the sheep. With experience the technical problems have been overcome and the investigators can now achieve a reliably manipulated pregnant sheep preparation in a consistent manner. They have used the electromagnetic flow transducers on a common uterine artery of the pregnant horn and the intra-abdominal fetal umbilical vein in addition to cannulation of multiple vessels to obtain the data which they accumulate. The pregnancy is not interrupted and can be expected to continue for several weeks in a chronic nonstress nonanesthetized state. This has permitted the broadening examination of those factors which influence both uterine and umbilical blood flow in the near-term pregnant sheep. The presence of beta and alpha adernergic receptors in the placental circulation have been examined in various species. The presence of and the responsivity of fetal beta receptors in the sheep have been demonstrated by the use of isoproterenol to either mother or fetus. This model has been used to test the effect of a number of drugs which are used in pregnant women and have not been evaluated in terms of effect on the uterus and the fetus. Using the techniques which this group has applied, they have looked at the effect on blood flow to the uterus and fetus as well as the effect on blood gases and certain metabolites. This becomes a very important system to apply since many of these drugs given to the pregnant female have not been tested in any way relevant to the clinical situation. The renin-angiotensin system has also been looked at in the pregnant animal. In the investigations relating to this system, the low sodium diet was imposed and it was very striking that the use of low sodium remarkably diminished fetal survival compared to survival on normal sodium diets.

Laboratory of Molecular Genetics - Accomplishments

1. Studies were directed towards the mechanism by which a virus, the bacteriophage lambda, inserts its chromosome into the chromosome of its host and how this intracellular virus nucleic acid is incorporated into the infectious particle. Using the powerful genetics selection techniques that have been developed in this laboratory, a large number of mutants defective in their ability to integrate and excise viral genetic information have been obtained. These genetic lesions have been assessed by genetic and electron microscopic techniques, and careful mapping studies have shown that three critical genes, att, int, and xis genes involved in the attachment, integration, and excision of the virus genome, are compactly located on a region of the viral chromosome not more than 1500 bases in length. The enzyme encoded by the int gene has been partially purified in collaborative studies, and product of int promoted recombination has been visualized.

The bacteriophage lambda has been modified for use as a safer vector for the formation of recombinant DNA molecules. This phage should be of enormous value to a host of scientists whose research will now turn to the formation of recombinant DNA molecules utilizing DNA from prokaryotic organisms in combination with that of higher organisms.

2. Studies were also directed towards the regulatory mechanisms which control an array of diverse biochemical and physiological activities in E. coli. As reported in previous years, work has been directed by this group toward understanding one of the broadest control processes yet discovered in bacterial cells which exerts a pleiotropic regulatory effect upon the expression of approximately 50% of the bacterial genome. This regulation is governed by guanosine tetrophosphate whose formation is determined by the rel gene.

Other studies have resulted in the construction of bacterial episomes containing short regions of the ribosome gene, encoding the ribosomal promoter (origin of transcription) site.

3. Studies were directed towards understanding the sequence of the actions which are involved in the synthesis, processing, and degradation of messenger RNA. Specifically, the role of ribonuclease III, a ribonuclease which attacks double stranded RNA has been examined in detail. This enzyme is known to duplicate the specific processing steps required for the production of ribosomal RNA, has also been found to produce specific cleavages in RNA derived from adenovirus and vesicular stomatitis virus, as well as to produce 18S and 28S size RNAs from ribosomal RNA precursors derived from Notophtalmus viridescens.

4. Studies have been directed towards understanding the assembly of RNA tumor viruses in a system which uncouples the synthesis of murine leukemia virus RNA from the terminal steps in viral maturation. It has been

discovered that viral particles produced under these conditions lack the high molecular weight 70S RNA genome and appear to contain only 8S and 4S RNA species. These virions have been shown to be identical to genomic RNA-containing virions with respect to their structural proteins. This indicates that the viral genome is not required to direct the assembly of viral proteins into viral particles. It has been shown that the structural proteins are synthesized de novo in the absence of viral RNA synthesis, leading to the conclusion that infected cells contain stable viral messenger RNA molecules.

5. The mechanism by which the virus gains control of the eukaryotic host biosynthetic machinery and towards developing the electron microscope as a tool for use in macromolecular analyses: This approach has made use of several diverse viral systems including polio- and adenovirus. Work with the poliovirus has led to the isolation and partial characterization of an intermediate in the initiation stage of poliovirus mRNA translation. Inasmuch as polio is translated to the exclusion of host mRNAs, it is the hope that further studies of this intermediate will yield important information about virus usurpation of host biosynthetic properties.

6. The control of eukaryotic gene expression has been studied in cultures of human cells infected with human adenovirus. The time course of production of early viral proteins likely to represent control elements of gene expression has been measured in the infected cells. Furthermore, messenger RNA coding for individual viral proteins has been extracted from these cells and has been visualized in the electron microscope. A new technique has been employed to show that nuclei of infected cells contain the large RNA that comprises virtually the entire viral DNA, whereas the cytoplasmic messenger RNA classes are much shorter and hybridize to defined points of the viral genome, according to the map position of the encoded gene functions.

7. The processes involved in cellular differentiation using the embryonic chick lens as a model system: These studies, which have quantitated the delta-crystallin mRNA during the process of lens cell differentiation from the very earliest phases of lens induction to terminally differentiating lens fiber cells, have shown that the delta-crystallin genes are not amplified during differentiation and have provided evidence that transcriptional activity is responsible for the expression of these genes.

8. The regulatory pathway effecting antibody synthesis at the cellular level: These studies have been directed toward delineating the role of (thymus-derived) T-cells of both the helper and suppressor types. A streptococcal exotoxin (SPE) has been shown to inhibit preferentially an activity thought to be controlled by suppressor T-cells. In fact, this observation lends strength to the notion that two populations of T-cells exist, one of which has a helper function, the other a suppressor function. SPE appears to have no effect on the helper function. The mechanism of interaction of SPE with T-cells will be investigated during the next year.

9. In the regulated expression and organization of two sets of mammalian genes, those corresponding to globin and those corresponding to

immunoglobulin: These studies have been based upon much previous developmental work which resulted in a technique for the isolation and assay of specific genetic sequences. Most recently, the process of erythrodifferentiation has been analyzed in considerable detail using cultured erythroleukemic cells. While it was previously shown by this laboratory that globin mRNA appears first as a function of erythrodifferentiation, this sequence of events has been shown to be composed of the sequential expression of the alpha, and then the beta globin genes. The sequence recapitulates oncological gene expression. It was shown that with butyric acid and gene replication, DNA synthesis and cell division are not required for expression of the globin genes.

Further studies of the origin of the immunoglobulin gene diversity strongly suggest that the variable region of the immunoglobulin mouse lambda chain is represented but few, possibly one time in the mouse genome. This finding lends further support to the notion that immunoglobulin diversity arises through a process of somatic mutation in which specific regions of the immunoglobulin genome undergo highly specific genetic alterations.

Behavioral Biology Branch - Achievements

1. Neurobiology: The cellular basis for synapse formation and long-term modulation of synaptic function are major areas of interest in this section. A major advance in approaching these problems has been made this year in demonstrating that neurons from continuous cell lines were capable of forming synapses with skeletal muscle. They have shown that these synapses are cholinergic and have many of the characteristics of immature synapses in vivo. The hybrid cells being studied have been shown to possess a number of types of receptors of neurobiologic interest and show electrical responses to dopamine, morphine, acetylcholine, prostaglandin, and serotonin.

A prototypic anesthetic agent, pentobarbital, has been shown to have selective effects on neuronal responses elicited by glutamate, an excitatory agent and gamma-aminobutyric acid, an inhibitory agent. Glutamate elicited depolarization of the neuronal membrane is decreased or abolished while GABA elicited hyperpolarization is prolonged and converted into a slight depolarization.

It was found that different nerve cells utilize different ionic mechanisms in producing active potentials; dorsal root ganglion cells develop calcium action potentials in part while spinal cord cells have action potentials solely dependent on sodium. The endoplasmic reticulum of neurons appear to serve as calcium sequestering site, and sub-surface cisterns in some nerve cells may serve as a means by which surface membrane events (synaptic or action potentials) may couple into this intracellular membrane-bounded system.

Total tissue RNA has been hybridized with unique DNA sequences as an index of diversity. Rats raised in enriched environment exhibit 20-30% greater brain RNA diversity than do rats raised in an impoverished environment. No change is found in liver RNA diversity.

II. Section on Functional Neurochemistry: The major focus of this section is to study peptides and proteins which are involved in intercellular communication. Because of the intrinsic heterogeneity of nervous tissue, a significant effort has been made in the area of microanalytical technology, so that individual neurons and small regions of brain can be studied biochemically as well as electrophysiologically.

Using the model system of identifiable neurons from the ganglia of molluscs, they have isolated a cholinergic prototype neuron as well as the bag cells which are typical peptidergic neurosecretory cells. The neurosecretory cells contain a peptide which acts as an anti-diuretic hormone, while the bag cell peptide induces egg-laying in the animal. The biosynthesis, axonal transport, and regulation of these peptides in these neurons has been intensively studied. The following observations can be made: (1) the peptide hormones are first synthesized as protein precursors which then undergo post-translational cleavage; (2) this processing of the precursor appears to occur in the Golgi as well as in the neurosecretory granules; (3) processing can occur intragranularly during axonal transport; (4) the rate of clearance of newly synthesized peptides is directly correlated with spike frequency; and (5) synaptic (dopamine) or behavioral input to the neuron can regulate synthesis and transport of the peptide.

The neurohypophyseal peptides (oxytocin and vasopressin) have been studied in rats, and synthesis in the hypothalamus as protein precursors and conversion during axonal transport has been demonstrated. The amphibian pars intermedia is another peptide releasing system uniquely suited for regulatory studies. In the toad the release of MSH is regulated by inhibitory control via dopamine, whereas in the chameleon the control is excitatory through serotonin. Attempts are being made to localize peptide hormones, (e.g., gastrin, ACTH, glucagon, etc.) in various brain regions using radioimmunological techniques.

Studies on the model system using the giant axon squid have shown that large proteins (> 200, 00 daltons), are synthesized by Schwann cells and transferred into the giant axon. This is the first unequivocal demonstration of macromolecular transfer between glia and neurons, in situ.

III. Section on Brain and Behavior: Neurophysiological studies of central auditory mechanisms in squirrel monkey utilizing species typical vocalizations as stimuli have been continued during the past year. Two substantial bodies of data have been collected during the past year: (1) an examination of 300 cells in the dorsolateral frontal cortex has been analyzed. A relatively low percentage of these cells (30%) were found which responded to local simuli, and the nature of timing of such responses suggests that this area of the brain does not contribute to the processing of vocal stimuli at the level higher than that found in the temporal auditory areas.

Examination of the characteristics of cortical regions in the temporal lobe which surround the primary auditory projection have been studied during the past year. A sample of 175 cells within the temporal gyrus and situated at varying distances from the primary projection have been studied. It is clear that the secondary areas have lower overall responsiveness

(65% as opposed to 90%) and contain more cells which are highly selective to one or a few vocalizations.

Laboratory of Biomedical Sciences - Accomplishments

1. Section on Developmental Enzymology: This group is concerned with the purification and characterization of placental enzymes, pregnancy-associated enzymes in maternal plasma, and fetal enzymes.

In a study of the control mechanism of gene expression in choriocarcinoma cells, alkaline phosphatase was found to be inducible by a variety of physiological and nonphysiological agents such as dibutyryl cyclic AMP, 5-bromodeoxyuridine, and inhibitors of DNA synthesis. The induction required both RNA synthesis and protein synthesis.

Glycogen synthetase from choriocarcinoma cells was found to resemble in many respects the enzyme from normal placenta, but it does have a K for glucose-6-P which is 5 to 10 times greater than the K for placental enzyme; a unique regulatory mechanism for D-form glycogen synthetase, through activation of manganese has been discovered; placental alkaline phosphatase has been proven to be a phosphoprotein phosphatase, one substrate of which is glycogen synthetase; multiple forms of cyclic AMP-independent phosvitin kinase have been separated and characterized.

In the area of amino acid metabolism, two enzymes have been discovered which mediate the first steps in the alternate route for the metabolism of intermediates of lysine degradation. This new route provides the basis of a model that may give insight into the biochemistry of several recently discovered defects in the intermediary metabolism.

2. Studies in the neuroendocrine mechanisms in two general areas of mammalian physiology - the pineal gland and the pituitary gland. Results indicated that the regulation of the enzyme which governs melatonin synthesis, N-acetyltransferase, is strongly influenced by temperature. This suggests that in addition to being a neuroendocrine phototransducer, the pineal gland may also be a neuroendocrine thermotransducer. Studies have provided evidence that during times of stress the pineal gland is not adrenergically stimulated substantially by circulating catecholamines, because the pineal nerves take up catecholamines and thus preclude the accumulation of these compounds in the pineal perivascular space. The method for culturing pineal cells has been developed. Using this system, the adrenergic-cyclic AMP regulation of pineal N-acetyltransferase activity has been studied.

The Section on Intermediary Metabolism has studied mental retardation through analogs of specific chemical agents and enzyme inhibitors, investigated the biosynthesis and degradation of macromolecules in neural tissues, studied the mechanism of hydroxylation reactions of neural interest, and has engaged in studies of the mechanism of action of nerve growth factor.

During the past year, most of the effort has been spent in developing the studies related to nerve growth factor. It has been demonstrated that changes in cyclic AMP levels are produced by the addition of nerve growth factor to superior cervical ganglia in organ culture and to isolated vesicles from adrenal medulla. It has been shown that the addition of dibutyryl cyclic AMP to ganglia produces an increase in tyrosine hydroxylase levels similar to, but not large as, that found upon addition of nerve growth factor.

Social and Behavioral Sciences Branch - Accomplishments

This branch is concerned with the interaction of the mother, infant, and father in relation to early development. It is only recently that they have become involved in assessing the paternal role in this interaction and have found a distinctly positive correlation. They have noted a significant relationship between the mother's psychological well-being during pregnancy and the neonatal characteristics of the infant. This is to be taken in the context that such women are likely to get less analgesia and anesthesia during labor and delivery than women less calm. They have shown that the mother acts more negatively towards the irritable girl infant than a boy infant, whereas in the father the reverse is true. On the other hand, the infant's alertness and motor maturity are associated with the father's report of esteem for his wife in the mothering role and less tension and conflict in the marriage. This group has also attempted to develop methods to measure motivation to master the inanimate environment in one-year-olds. They have developed three types of measures: tasks requiring problem-solving, those involving the practice of emerging skills, and tasks which provide opportunities to produce effects and obtain feedback from the environment.

Previous studies showed significant relationships between the six-month environment and the cognitive development of the infant in low socioeconomic families. A study of the middle class families by the same technique showed major differences. The mother from the latter group gave their infants their tactful, visual, and auditory stimulation; they played with them more, smiled more frequently at them, and they are more responsive to their positive vocalizations. They also provide their infants with a greater variety of play materials. These findings have implications for intervention programs.

A study of the components related to infant attachment has been undertaken. It has been found that variations in separation protests are not indicative of the strength of the mother-infant bond. It has been found that the distress registered in the presence of a stranger is related to the separation response. It would appear that to answer the question asked, new methodology must be developed which would appear to have to be framed in terms of the level of stress which the infant can tolerate.

NICHD Annual Report
July 1, 1975 through June 30, 1976
Social and Behavioral Sciences Branch

Summary of Research Activities

During the past several years research in the Social and Behavioral Sciences Branch has been concerned mainly with analysis of early experiences and their impact on early development. A major aim has been to develop methods to study the infant in his natural environment and to differentiate the concept of early experience into its principal components. The empirical findings of our studies and the concepts that emerged from them have led us to view early experience as a complex interactive system that includes the father as well as the mother, the infant and some aspects of the inanimate environment. Currently two studies are in progress which are attempting to look at the environment from this more complex perspective. Our previous studies also heightened our awareness of the many facets of infant temperament, the varied repertoire of infant responses and initiatives to people, the richness of his behavior that goes beyond simple cognitive functions. We are especially interested in the infant's motivation to act on and to master his inanimate environment. We are currently attempting to conceptualize and develop behavioral measures of these more subtle aspects of infant functioning. We are also trying to understand the origins of the infant's temperament in his prenatal and perinatal experiences.

In regard to the origins of infant temperament, we have found significant relationships between the mother's psychological well-being during pregnancy and the neonatal characteristics of the infant. This is not a simple relationship, however, since women who adapt well during pregnancy receive lesser amounts of analgesia and anesthesia during labor and delivery. The infants of these women in turn, as assessed neonatally on the Brazelton Scales, are more mature motorically and more alert. We also find that the infant's early characteristics have significant effects on maternal behavior. There are significant relationships between the neonate's temperamental characteristics and the mother's behavior towards the infant at six months. For example, the infant's alertness at 3 days is positively related to the mother's expression of positive affect, to the amount of time she plays with him and the variety of play materials she provides for him. For girl infants there are also significant negative relationships between their irritability neonatally and the mother's positive affect and the time she spends in play. Mothers show less positive affect towards irritable girl infants and play less with them. These findings provide empirical support for the infant's contribution to the mother-infant interaction and they indicate the need for interactive models for early relationships.

Complementing our findings on the infant's influence on the mother-infant relationship, we have also found that the baby's temperamental characteristics selectively affect the father-infant interaction and the husband-wife relationship when the infant is four weeks of age. When the male neonate is more irritable the father is likely to describe stronger negative affect toward him. On the other hand, the infant's alertness and motor maturity are associated with the father's report of esteem for his wife in the mothering role and less tension and conflict in the marriage. Both of these variables, in turn, are related to the mother-infant relationship. These findings support the concept of the father as an integral part of the early environment. His interaction with the infant is influenced by the baby's temperament, and his behavior affects mother's feelings and behavior towards the infant as well. In contrast to previous socialization models which have largely ignored both the infant's temperamental characteristics and the contribution of the father, this research gives an empirical basis for conceptualizing the early environment as an interactive, three-person system. Our goals are to derive a more meaningful conceptualization of a three-person interactional system and to develop more sensitive methods to describe interactional processes in the nuclear family.

Our view of the infant as an active force in his environment has stimulated an interest in other aspects of infant development than purely cognitive functions. It has led to attempts to develop methods to measure motivation to master the inanimate environment in one year olds. We have developed three types of measures: tasks requiring problem-solving, those involving the practice of emerging skills, and tasks which provide opportunities to produce effects and obtain feedback from the environment. We believe that these behaviors come to be associated with the infant's sense of competence and his feelings that he can exert some degree of control over his environment. The first analyses have been concerned mainly with the adequacy of the tasks and the measures derived from them. We have analyzed the interrelationships among the measures and their relationships to more traditional indexes of early development such as the Bayley Scales. Measures of persistence on the three types of tasks are only moderately interrelated, indicating that there are different aspects of cognitive-motivational functioning. On the other hand, we find that measures of persistence across sessions on similar tasks are more highly related. There is also a significant relationship between the overall persistence score on the three types of mastery motivation tasks and the Bayley Mental Developmental Index. These findings indicate that the infant's motivation to produce effects, to persist at problem solving and to master emerging skills are closely intertwined with his cognitive development. Moreover, persistence is not only essential for the solution of difficult problems, it is also important in perfecting skills and in obtaining feedback from the environment. Many of the tasks and the measures derived from them have proved useful in distinguishing individual differences in infants. A next important step is to try to understand the origins of these motivational differences. We plan to explore the antecedents of these behaviors by analyzing their relationships to 6 month environmental variables.

In a previous study of infants and mothers from the inner city, families of low socioeconomic status, we found many significant relationships between the six month environment and the cognitive development of the infant. Inasmuch as these findings had significant theoretical and practical implications, we thought it important to examine these relationships in other cultural contexts. Therefore, we replicated the earlier study with a sample of middle class infants and mothers using the same home observation technique and the same measures of infant development. Comparisons of the two samples on the major caretaking variables have been made. We find a number of significant differences between these two samples. The mothers in the higher socioeconomic group give their infants more tactile, visual and auditory stimulation, they play with them more, smile more frequently at them, and they are more responsive to their positive vocalizations. They also provide their infants with a greater variety of play materials, and these materials are more responsive and more complex. These findings have implications for intervention programs. They also raise questions about the long term effects of relative deprivation and enrichment. Moreover, there are interesting theoretical questions regarding the effects of the level of stimulation on the statistical linkages between the environment and infant development, questions which have implications for identifying optimal levels of stimulation.

Another project was concerned with analyzing a specific aspect of early experience, the stimulation infants receive through social play in their homes at one year. There is a significant relationship between the variety of cognitive activities in social play and the Bayley Mental Developmental Index. This environmental variable is also related to several clusters derived from the Bayley, i.e., Language and Perceptual Discrimination, and with some motivational measures, persistence in problem solving and persistence in practicing emerging skills. These findings extend our understanding of early cognitive development by showing that the experiences which infants have in social play in the home environment are significantly related to cognitive functioning during the sensorimotor period.

In recent years considerable attention has been given to the infant's earliest social relationships, particularly his attachment to his mother. A large number of techniques and many different indexes have been used to study this early focused relationship. There are many theories of the origins of attachment, some maintaining that it is largely instinctive, rooted in the human infant's helplessness, and therefore essential for his survival. Others hold that the infant's tie to the mother is learned, and that it evolves through the infant's earliest interactions with people who provide nurturance.
Before one can resolve such basic questions, important methodological and conceptual issues must be resolved. A methodological study of attachment is in progress. This study is designed to compare data obtained through several different approaches: observations in structured situations in the laboratory, observations in similar situations in the home, interviews with the mother regarding the infant's response to strangers and to separation from her. The most commonly used measure of attachment is the baby's protest on being separated from the mother. Our data suggest that variations in separation protest are not indicative of the strength of the mother-infant bond. The degree of protest after separation appears to be very much influenced by the stress the infant is experiencing in the immediate situation preceding the separation. In regard to another common index of attachment, reactions to strangers, we have looked at several factors related to the infant's expression of fear of strangers: general irritability, a tendency to show distress in everyday separations from the mother, exposure to few unfamiliar persons early in infancy. Both the temperamental charastic of irritability and the tendency to show separation distress in common everyday situations were significantly related to stranger distress. Although a history of exposure to few adults in infancy was not significant by itself, this variable has some influence in conjunction with the two other factors. The findings of this study emphasize that it is not meaningful to attempt to characterize infants in terms of intensity of attachment. It appears that the degree of reaction to strangers and the intensity of separation distress vary with the infant's general tolerance for stress. This study, addressing itself to important methodological issues, is a first step in clarifying the process and underlying mechanisms of attachment.

Members of the Branch have served on committees of the N.I.H. and of national professional organizations and have been members of editorial boards and editorial consultants to the major child development journals. They have participated in a variety of ways in the meetings of professional organizations as invited speakers, in planning programs, as reviewers of papers and in presenting their own research.

Dr. Juarlyn Gaiter has served as a member of the Day Care Alliance Committee of the National Association for the Education of Young Children, has been a program committee member of the Black Caucus of the Society for Research in Child Development, and of the National Council of Black Child Development. Dr. Gaiter presented an invited paper on "Maternal separation in infancy and early childhood" at the meetings of the National Association for the Education of Young Children. She participated in a panel on "Surrogate Parenting" sponsored by the Washington Area Child Development Seminar. She also presented an invited paper, "Life Cycle in Perspective" at a forum on human ecology at Howard University.

Drs. Barbara Anderson and Robert Harmon have served as consultants to the Vulnerable Infant Project of the Mental Health Study Center of the National Institute of Mental Health. Dr. Harmon has been a member of the Film Archives Committee of the Society for Research in Child Development. During the past year he has been a clinical instructor of Psychiatry and Behavioral Science at the George Washington School of Medicine. At the annual meeting of the American Academy of Child Psychiatry, Dr. Harmon presented a paper written by Drs. Harmon, Morgan and Klein, entitled "Normal variations in infant's negative reactions to unfamiliar adults." He has also given a series of lectures on the first year of life to the Departments of Psychiatry at Children's Hospital and at the Georgetown University School of Medicine.

Dr. Robert Klein has served as a member of the National Institute of Child Health and Human Development Task Force on Dyslexia. He has also been a consultant to the Alta Vista Day Care Center and a consultant on research to the Department of Pediatrics, Soroka Medical Center, Beer-Sheba, Israel, regarding a study on calorie malnutrition. With Ms. Joan Durfee, Dr. Klein was the co-author of a paper, "Patterning of infants' prior separation experience in relation to later separation reaction," which was presented by Ms. Durfee at the Southeastern Conference on Child Development. Dr. Klein also gave an invited address in the lecture series on Studies in Infancy at the Kennedy Institute of Johns Hopkins University. At the annual roundtable of the Maryland Day Care Association he also served as discussion leader for a panel on Discipline and Other Problems in Family Day Care.

Dr. George Morgan has been a consultant to the Canada Council, reviewing research proposals on early intervention to facilitate infant development.

Dr. Frank Pedersen continues to serve as a member of the N.I.H. Day Care Committee and the National Institute of Child Health and Human Development Clinical Review Committee. He has been an editorial consultant for the journals, Child Development and the Psychological Bulletin. Dr. Pedersen presented a paper, "Mother, father and infant as an interactive system" on a symposium at the annual meetings of the American Psychological Association. At the meetings of the Southeastern Conference on Human Development Dr. Pedersen presented a paper, "A methodology for assessing parental perception of infant temperament" (in collaboration with Barbara Anderson and Richard Cain).

Dr. Kay Standley is serving on the Board of Convention Affairs of the American Psychological Association and is a member of the APA Task Force on Continuing Education. She is also an Editorial Consultant to the Journal of Consulting and Clinical Psychology. She continues to serve as the Women's Program Coordinator of the National Institute of Child Health and Human Development. Dr. Standley presented two invited addresses, one entitled "Events of childbirth," at the Third National Maternal-Infant Life Conference; the second on "Women in Scientific Professions" at Utah State University. Dr. Standley also presented an invited paper, "Sources of variation in the behavior of normal newborns: Consequences in the Etiology of Behavioral Dysfunction" at the annual meeting of the Eastern Psychological Association. At the annual convention of the American Nursing Association, she presented an invited paper, "The psychological experience of labor" on a symposium of "Parenting."

Dr. Leon Yarrow continues to serve on the Board of Trustees of the Easter Seal Research Foundation and is a member of the advisory Committee of the Vulnerable Infant Project of the Mental Health Study Center of the National Institute of Mental Health. He was a research consultant to the Canada Council and the Grant Foundation. Dr. Yarrow continues to serve on the editorial board of the Merrill Palmer Quarterly Journal of Behavior and Development and he has been editorial consultant to Child Development, the Child Development Monographs and Human Development.

At the biennial meeting of the International Society for the Study of Behavioral Development at the University of Surrey in Guildford, England, Dr. Yarrow presented a paper, "A conceptualization of father influences and its implications for an observational methodology," prepared by F. Pedersen, L. Yarrow and B. Anderson. He chaired and was a discussant on a symposium, "Effectance motivation: A concept in need of measures" at the meetings of the Southeastern Conference on Child Development. On this symposium papers were presented by several members of the Social and Behavioral Sciences Branch: "Aspects of mastery motivation and cognitive functioning" by George Morgan, "Cognitive play experiences and 13-month-old infant experience with objects" by Juarlyn Gaiter, and "Free play and effectance motivation" by Kay Jennings.

Publications Not Directly Related to any Specific Project:

Emde, R. N., Gaensbauer, T. J., & Harmon, R. J. Emotional expression in infancy: A bio-behavioral study. Psychological Issues, 10: 1976, in press.

Goodson, F. E., & Morgan, G. A. The evaluation of scientific theories. In M. H. Marx & F. E. Goodson (Eds.), Theories in Contemporary Psychology (2nd edition). New York: Macmillan, 1976, pp. 286-299.

Goodson, F. E., & Morgan, G. A. On levels of psychological research. In M. H. Marx & F. E. Goodson (Eds.), Theories in Contemporary Psychology (2nd edition). New York: Macmillan, 1976, pp. 394-407.

Harmon, R. J. Review of Green, R. Sexual identity conflict in children and adults. Child Development Abstracts and Bibliography, 49: 342, 1975.

Harmon, R. J., & Emde, R. N. Review of Anders, T. The infant sleep profile. Sleep Review, 127: 17, 1975.

Jennings, K. D. People vs. object orientation in preschool boys and girls. Educational Resources Information Center, ED 111 502 (Bethesda, Md.: NICHD, 1975).

Standley, K. Men on the move. Contemporary Psychology, 20: 825-826, 1975.

Yarrow, L. J. Study of behavioral development. A review. American Scientist, 63: 357-358, 1975.

Yarrow, L. J. The mechanisms of deprivation. Comtemporary Psychology, 21: 126-127, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00002-04 SBS

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Early Environmental Influences on Cognitive and Personality Development: A Replication

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. J. Yarrow	Chief, Social & Behavioral Sci. Br.	SBS	NICHD
OTHER:	R. P. Klein	Research Psychologist	SBS	NICHD
	F. A. Pedersen	Research Psychologist	SBS	NICHD
	J. T. Durfee	Research Psychologist	SBS	NICHD
	M. W. Fivel	Research Assistant	SBS	NICHD
	J. L. Gaiter	Staff Fellow	SBS	NICHD
	R. L. Cain	Research Assistant	SBS	NICHD
	N. F. Gist	Research Assistant	SBS	NICHD

COOPERATING UNITS (if any)

Pediatrics and Obstetrical Service, National Naval Medical Center

LAB/BRANCH

Social and Behavioral Sciences Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.35	.40	.95

SUMMARY OF WORK (200 words or less - underline keywords)

This is a replication with a middle class sample of 49 infants of a study previously carried out with a sample composed largely of lower socioeconomic infants and families. Data on environmental variables are obtained through time-sampling observations in the homes. Measures of infant development are obtained from the Bayley Scales and through a structured test designed to measure exploratory behavior and preference for novel stimuli. In the earlier study significant relationships were found between specific environmental variables and specific aspects of infant development at 6 months. For example, kinesthetic stimulation by the mother was significantly related to the Bayley Mental Development Index and to measures of Goal Directedness, Object Permanence and Social Responsiveness. We are replicating this study in order to determine whether similar relationships will hold in patterning of stimulation.

Preliminary analyses indicate that there are a variety of differences in maternal handling of infants in these socioeconomic groups.

Project Description:

Objectives: In a previous study (Z01.00001) with a sample composed largely of infants and mothers of low socioeconomic status, significant relationships were found between specific early environmental variables and specific aspects of infant development at 6 months. For example, infants whose mothers were responsive to their distress signals tended to show better developmental progress, i.e., to obtain higher Mental and Psychomotor Developmental indexes on the Bayley Test. Kinesthetic stimulation by the mother was also related to measures of Goal Directedness, Object Permanence and Social Responsiveness. Because of the theoretical significance of these findings, we are replicating the previous study with a middle-class sample.

Methods Employed: The sample consists of 49 infants, 25 boys and 24 girls and their mothers, of middle socioeconomic status. Many of these infants and mothers were first studied as neonates in the research on bidirectional influences, then at 5 to 6 months in this study and again at 12 months.

Data on environmental variables were obtained through time-sampling observations in the natural environment of the home when the infants were 5 months of age. Measures of infant characteristics were obtained by means of the Bayley Scales of Infant Development and a structured situational test designed to measure exploratory behavior and preference for novel stimuli. In addition to the Mental Developmental Index and the Psychomotor Developmental Index, eight clusters of items were derived from the Bayley Scales: Gross Motor Development, Fine Motor Development, Social Responsiveness, Language, Goal Directedness, Secondary Circular Reactions, Reaching and Grasping, and Object Permanence.

Major Findings: Several analyses have been completed, others are in progress. We have compared the environmental variables and the cognitive and cognitive-motivational characteristics of the infants in this sample of middle socioeconomic status families with our previous sample composed largely of lower socioeconomic families. We find a number of significant differences between these two samples on environmental variables. The mothers in the higher socioeconomic group give their five month infants more tactile, visual and auditory stimulation, they play more with them, smile more frequently at them, and they are more responsive to their positive vocalizations. Similarly, the character of the inanimate environment differs for the two groups. The middle socioeconomic infants are provided with a greater variety of play materials, and these materials are more complex and more responsive. We have not yet completed multivariate analyses of these parameters of stimulation, nor have we examined interactions among the variables or the correlations between the environmental and dependent variables. The fact that this sample is significantly higher on many aspects of environmental stimulation than the sample studied earlier raises some important research questions. One question is whether different levels of stimulation affect the nature of the statistical linkages between environmental variables and measures of infant functioning. This question is now being examined in further statistical analyses.

Significance to Biomedical Research and the Program of the Institute: Conclusions with significant theoretical and practical implications are often based on a single study in which the sample is restricted to one sub-cultural group. If we are to make meaningful generalizations about the significance of specific aspects of early experience, it is necessary to examine these relationships in a variety of environmental contexts, with samples that might differ in level and patterning of stimulation.

Proposed Course of Project: In the coming year, further data analyses will be carried out and the findings will be reported in professional journals. A followup study of these infants is planned when they are 31/2 years of age to investigate whether there are longterm effects on functioning of the early environment.

Publications:

Yarrow, L. J., & Pedersen, F. A. The interplay between cognition and motivation in infancy. In M. Lewis (Ed.), Origins of Intelligence: Infancy and early childhood. New York: Plenum Publishing Corporation, 1976.

Yarrow, L. J. Early experience: Maternal deprivation. In B. Wolman (Ed.), International Encyclopedia of Neurology, Psychiatry, Psychoanalysis and Psychology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00003-05 SBS

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

An Observational Study of Father-Infant Interaction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F. A. Pedersen	Research Psychologist	SBS	NICHD
OTHER:	B. J. Anderson	Staff Fellow	SBS	NICHD
	R. L. Cain	Research Assistant	SBS	NICHD
	B. C. Marcus	Research Psychologist	SBS	NICHD
	N. F. Gist	Research Assistant	SBS	NICHD

COOPERATING UNITS (if any)

Pediatrics and Obstetrical Service, National Naval Medical Center
Parent and Child, Childbirth Education Associates

LAB/BRANCH
Social and Behavioral Sciences Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.60	1.6	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are to obtain descriptive data on father-infant interaction in natural settings and to analyze the father's influence on the infant's social, cognitive and motivational development. With a sample of first-born 5-month-old infants, assessments include observations of father-infant, mother-infant and husband-wife interaction, a Q-sort technique to measure parental perceptions of infant temperament, parental interviews, and the Bayley Scales of Infant Development. A major goal is to develop a conceptual model for understanding the distinctive experiences provided by the father and his contribution to the early environment.

Project Description:

Objectives: To obtain descriptive data on the father-infant relationship in natural settings and to analyze the father's influence on the infant's social, cognitive and motivational development. This study is attempting to identify some of the distinctive experiences provided by fathers in the infancy period and to ascertain his impact upon the mother's relationship with the infant. A major goal is to develop a conceptual model for understanding paternal influences as a part of the early environment.

Methods Employed: The original sample for this project consisted of 39 middle-class fathers and their first-born infants (a subgroup of the sample in Project Z01.00004). When the infant was four weeks old the father was interviewed, and at five weeks an observation was conducted in the home focusing on the father's interaction with the infant.

In an extension of this project, a new sample is being obtained of forty to fifty middle-class couples all of whom have participated in a childbirth preparation class. Observational procedures developed in a previous study have been refined and extended to permit the description of father-infant, mother-infant and husband-wife interaction in the same setting. Two observations are made of mother-father-infant interaction and one of mother-infant interaction when the infants are five months of age. To complement the observations both parents are interviewed to obtain background information regarding the pregnancy and delivery and to assess the larger context of parent-infant involvement. In addition, each parent's perceptions of the infant's temperament are obtained by a Q-sort technique. The Bayley Scales of Infant Development are administered when the infant is 6 months old. Clusters of infant cognitive, social and motivational characteristics developed from a previous study are used as dependent variables.

Major Findings: Analyses indicate that the baby's temperamental characteristics measured independently in the neonatal period selectively influence the father-infant interaction and the husband-wife relationship when the infants are four weeks of age. The father of a neonate who is more irritable is likely is a significant relationship between the neonate's alertness to describe stronger negative affect toward the infant. There and motor maturity and the husband-wife relationship at 5 weeks; these infant characteristics are associated with the father's report of greater esteem for his wife in the mothering role and less tension and conflict in the marriage. Both of these variables, in turn, are related to aspects of the mother-infant relationship. These findings support the concept of the father as an integral part of the early environment. His interaction with the infant is related to the baby's temperament, and the father's behavior appears to affect the the mother as well. These findings which are evident only with male infants, are consistent with previous research which showed that a premature infant is associated with strain in the husband-wife relationship. This study extends such findings into the normal range of infant variation and marital adjustment. A report of the results, "Mother, Father and Infant as an Interactive System" was presented at the annual meetings of the American Psychological Association, September, 1975.

Recognition that the father-infant interaction is influenced by the temperamental characteristics of the infant has led to the development of a technique for assessing parental perception of infant temperament for the new sample of 5 month old infants. A Q-sort method was developed, building upon previous research by Thomas, Birch, Chess and others. The technique overcomes some of the psychometric limitations in previous instruments of this nature, such as problems of response set and poor inter-item consistency. The technique lends itself to study of how objective characteristics of the infant are translated into parental perceptions and expectancies. One of the interesting issues which is being pursued in the sample is to determine the areas where the mother and father have congruent perceptions of the infant's temperament as opposed to areas where their perceptions are discrepant. A report of the methodology and preliminary findings regarding inter-item consistency was presented at the Southeastern Regional meeting of the Society for Research in Child Development.

One of the problems which has impeded progress in this area of research in the past has been a preoccupation with studying effects on the child by comparing children reared in such grossly different groups as father-absent and father-present families. The processes by which father effects occur have been left largely to speculation. This problem was discussed at greater length in an invited paper submitted to the journal, The Family Coordinator. Present research efforts are concentrated on process variables. In traditional theories of environmental influences, conceptualization of father influences, particularly in the infancy period, has received scant attention. As a result of our observations and drawing upon other research, some progress has been made in sharpening our concepts of father influences and in developing hypotheses of his effects early in the infant's life. A conceptual paper of paternal influences on early development was presented at the meeting of the International Society for the Study of Behavioral Development, Surrey, England in July, 1975.

Significance to Biomedical Research and the Program of the Institute: Most of our knowledge of early parental behavior is limited to the mother-infant dyad. The assumption that this is a closed system, relatively unaffected by the father, and that early paternal behavior is of no consequence has not been questioned on a theoretical or an empirical level. Therefore, the observational data on father-infant interaction will contribute to our understanding of another aspect of the infant's early environment.

Proposed Course of Project: Data collection on the sample of 5-month-old infants will be completed in 1976 and analyses of the multiple sources of data will begin. A symposium is being planned for the biennial meeting of the Society for Research in Child Development on conceptualizing the early environment from a family perspective.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00004-04 SBS

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Bidirectional Influences in Mother-Infant Interaction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. J. Yarrow	Chief, Social & Behavioral Sci. Br.	SBS NICHD
OTHER:	K. Standley	Clinical Psychologist	SBS NICHD
	R. P. Klein	Research Psychologist	SBS NICHD
	R. L. Cain	Research Assistant	SBS NICHD
	N. F. Gist	Psychology Technician	SBS NICHD

COOPERATING UNITS (if any)

Pediatrics and Obstetrical Service, National Naval Medical Center

LAB/BRANCH

Social and Behavioral Sciences Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.10	.85	.25

SUMMARY OF WORK (200 words or less - underline keywords)

This longitudinal investigation has several interrelated objectives: 1) to study the relations between the adaptation of the husband and wife during pregnancy, and the mother's experiences during labor and delivery; 2) to examine the relations to early infant temperament of the woman's prenatal adaptation and her experiences during labor and delivery; 3) to investigate the relations between the husband-wife relationship during pregnancy and the early mother-infant relationship; 4) to study the stability of infant temperamental characteristics; 5) to assess the extent to which maternal behavior is influenced by infant temperament.

The sample consists of 46 parents and their first-born infants, 24 boys and 22 girls. Only normal infants, free from complications of pregnancy and delivery were included in the study.

Data are being obtained through interviews during the last trimester of pregnancy; from the hospital records regarding labor and delivery; through administration of the Brazelton Neonatal Assessment Scale at 3 days and 4 weeks; through time-sampling observations of mother-infant interaction in the home at 4 weeks and at 6 months; and through interviews with the mother at 4 weeks.

PHS-6040
(12-75)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00004-04 SBS

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Bidirectional Influences in Mother-Infant Interaction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. J. Yarrow	Chief, Social & Behavioral Sci. Br.	SBS	NICHD
OTHER:	K. D. Jennings	Staff Fellow	SBS	NICHD
	R. P. Klein	Research Psychologist	SBS	NICHD
	R. L. Cain	Research Assistant	SBS	NICHD
	M. W. Fivel	Research Assistant	SBS	NICHD

COOPERATING UNITS (if any)

Pediatrics and Obstetrical Service, National Naval Medical Center

LAB/BRANCH

Social and Behavioral Sciences Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.10	.85	.25

SUMMARY OF WORK (200 words or less - underline keywords)

This longitudinal investigation has several interrelated objectives: 1) to study the relations between the adaptation of the husband and wife during pregnancy, and the mother's experiences during labor and delivery; 2) to examine the relations to early infant temperament of the woman's prenatal adaptation and her experiences during labor and delivery; 3) to investigate the relations between the husband-wife relationship during pregnancy and the early mother-infant relationship; 4) to study the stability of infant temperamental characteristics; 5) to assess the extent to which maternal behavior is influenced by infant temperament.

The sample consists of 46 parents and their first-born infants, 24 boys and 22 girls. Only normal infants, free from complications of pregnancy and delivery were included in the study.

Data are being obtained through interviews during the last trimester of pregnancy; from the hospital records regarding labor and delivery; through administration of the Brazelton Neonatal Assessment Scale at 3 days and 4 weeks; through time-sampling observations of mother-infant interaction in the home at 4 weeks and at 6 months; and through interviews with the mother at 4 weeks.

Project Description:

Objectives: This longitudinal investigation has several interrelated objectives: 1) to study the relations between the psychological and physical adaptation of the husband and wife during pregnancy, and the mother's experiences during labor and delivery, e.g., the ease or difficulty of delivery, the medication she is given during the birth process; 2) to examine the relations to early infant temperament of the woman's prenatal adaptation and her experiences during labor and delivery; 3) to investigate the relations between prenatal experiences, especially the husband-wife relationship during pregnancy and the early mother-infant relationship (at four weeks); 4) to study the stability (over the first six months of life) of infant temperamental characteristics assessed neonatally; 5) to assess the interaction between neonatal temperamental characteristics and maternal caretaking patterns in order to determine the extent to which maternal behavior is influenced by infant temperament.

Methods Employed: The sample consists of 46 parents and their first-born infants, 24 boys and 22 girls. Only normal infants, free from complications of pregnancy and delivery were included in the study. There were five phases of data collection:

1. The prospective parents were interviewed during the last trimester of pregnancy regarding their psychological and physiological adaptation to the pregnancy experience, the degree of solidarity between husband and wife, their expectations of the delivery, their feelings and anticipations about the unborn child.
2. Information was obtained from the hospital records regarding the labor and delivery, and the status of the infant at birth, e.g., Apgar score, birth weight.
3. Infant characteristics were assessed at 3 days and at 4 weeks with the Brazelton Neonatal Assessment procedure.
4. Time-sampling observations of mother-infant interaction in the home were made at 4 weeks and again at 6 months.
5. The mother was interviewed at 4 weeks regarding her feelings towards the infant and her view of his characteristics, and her satisfactions and dissatisfactions with the parenting role.

Major Findings: We find significant interrelationships among a number of variables: the mother's adaptation during pregnancy, the course of labor and delivery, parental characteristics, such as age and income. For example, we find significant relationships between the mother's adaptation during pregnancy and the amount of analgesia and anesthesia given in labor and delivery. Older, more highly educated and financially secure couples are more likely to have a satisfying pregnancy, to approach childbirth with eagerness and assurance and then to have less analgesic medication than their younger, more anxious counterparts. Many of these prenatal and perinatal measures relate to neonatal behaviors. The advantaged infants are more responsive and motorically mature and less irritable. Further data analysis is being directed towards an understanding of the relative contributions of prenatal and perinatal conditions on neonatal behaviors. No one set of variables seems to stand out as the most important determinant of infant temperament, rather a number of prenatal and perinatal measures are related to infant characteristics.

Other analyses point to the importance of infant temperamental characteristics for maternal caregiving behavior at 6 months. Significant relationships were found between the infant's visual orientation to people and the mother's expression of positive affect towards him (r=.49*), the amount of time she actively plays with him (r=.55**) and the variety of inanimate stimulation she gives him (r=.40*). These relationships are much stronger for boys (r=.66** with positive affect; r=.70** with play; r=.62* with variety of social stimulation and r=.55** with variety of inanimate stimulation). The relationships between the infant's irritability and these aspects of maternal behavior are negative; there are strong negative relationships between irritability in girls and mother's positive affect (r=-.65*) and play (-.57*); whereas there are essentially no relationships between the irritability of boys and the mother's behavior.

These findings suggest that infants who are socially oriented as neonates tend to elicit more positive affect from their mothers, their mothers play more with them, give them more varied social stimulation, and offer them a greater variety of inanimate stimulation. Mothers of highly irritable infant girls show less positive affect towards them and play less with them. The findings that neonatal infant characteristics are related to maternal behaviors at 6 months suggest that very early temperamental characteristics set in train patterns of interaction which influence the mother-infant relationship of time. Patterns of mother-infant interaction that have continuity over time may be established very early through the complex transactions between maternal and infant temperaments.

Significance to Biomedical Research and the Program of the Institute: In this investigation we are trying to go beyond the simple unidirectional model of looking at the impact of early experiences, we are analyzing infant and maternal behavior as part of a system of mutual regulation. This new perspective should give better understanding of the complex interactions between parental characteristics and expectations regarding the infant and infant temperamental characteristics.

* Significant at 5% level
** Significant at 1% level

Proposed Course of Project: Analyses of data will be completed during the coming fiscal year, and reports of the findings and their significance will be prepared.

Publications:

Standley, K., Klein, R. P., & Soule, A. B. Local-regional anesthesia during childbirth and newborn behavior. Science, 1975, 189, 572.

Standley, K., Soule, A. B., Copans, S. A. & Duchowny, M. S. Local-regional anesthesia during childbirth: Effect of newborn behaviors. Nursing Digest, 1976, 4, 27-28.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00005-03 SBS

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Consistency and Antecedents of Infant Attachment During the First Year

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. P. Klein	Research Psychologist	SBS	NICHD
	J. T. Durfee	Research Psychologist	SBS	NICHD
OTHER:	M. W. Fivel	Research Assistant	SBS	NICHD
	G. A. Morgan	Research Psychologist	SBS	NICHD
	C. A. Bennett	Research Assistant	SBS	NICHD
	R. J. Harmon	Research Associate	SBS	NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Social and Behavioral Sciences Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	.95	1.5

SUMMARY OF WORK (200 words or less - underline keywords)

This is primarily a methodological study of infant social behavior at one year. Measures of infant behavior in several situations and several settings were made. We are studying the relationships among these measures in order to address the conceptual issue of what are the most appropriate behavioral measures of mother-infant attachment. As part of this effort we are also studying reactions to strangers. In addition to the methodological question we plan to examine the antecedents of the distinctive tie between mother and infant. Approximately half the sample was observed at six months as part of Project Z01.HD0002-04. Several of the environmental variables observed at that time have been hypothesized to relate to later attachment, e.g., amount of kinesthetic stimulation and mother's responsiveness to baby's distress.

Project Description:

Objectives: This study has both methodological and substantive objectives. Previous studies on attachment have used a variety of techniques--interview with the mother, structured situations in the laboratory and varied indices of attachment. In this study, we are comparing data obtained through several different approaches: observations in the home, an interview with the mother, and observations of structured situations in the laboratory. Underlying this methodological quesstion is the issue of what are the most appropriate behavioral measures of this relationship.

A second objective is to study the antecedents of the distinctive tie between mother and infant. The relationship of several environmental variables studied at 6 months, e.g., amount of kinesthetic stimulation, mother's responsiveness to baby's distress, to measures of attachment at 12 months will be examined.

Methods Employed: The sample consisted of forty mothers and their twelve month old infants, 20 boys and 20 girls, with half of each sex being first and half later born. About half of the sample was seen at six months (Project No. Z01 HD 00002).

Two home observations of two hours duration, approximately a week apart were conducted within two weeks of the baby's birthday. Observations were made of selected mother and infant behaviors in a number of situations which were comparable to measures that had been used in laboratory studies of attachment behavior. The infant's reactions to such events as the mother entering the room, leaving the room, the appearance of the mother when the infant was crying were recorded. The infant's vocalizations, smiles, approach behaviors, cessation of crying were also recorded.

Following completion of the two home observations, the mother and infant were brought to a laboratory playroom for a series of observations a week later. The procedures included the following:

1. The infant's initial response to an unfamiliar adult, was observed from behind a one-way mirror. The experimenter approached the infant in a pre-determined, structured but natural manner. The mother was then asked to go through the same approach.

2. The mother was interviewed, using a semi-structured interview regarding selected aspects of her baby's social functioning in relation to herself, the baby's father, other familiar people, and strangers.

3. During the interview, the infant was provided with toys, and his exploratory behavior, his social initiative and

responses to the mother and interviewer were observed from behind the one-way mirror.

4. At the completion of the interview proper, the mother was asked to leave the room and the infant's reactions to being left with the interviewer were recorded. His reactions to the mother's return were also noted.

Major Findings: The bulk of the data analysis has been completed and papers are being prepared for presentation at professional meetings. A report on the findings concerning one of the central questions of the study, i.e., what are the most appropriate measures of the distinctive tie between mother and infant, is currently in preparation. The most commonly used measure of this tie is separation protest. The study was designed to enable us to determine the relationship between this measure and others which have been used. The results of our analyses indicate that variations in separation protest in a structured laboratory situation do not indicate differences in strength of the mother-infant bond. Rather, they appear to reflect the extent of stress the infant is experiencing just prior to the separation. This is shown by the significant correlations between separation protest and measures of distress made before the separation occurs.

A separate set of analyses has focused on factors which may influence individual differences in another index frequently used to define attachment, the infant's reactions to an unfamiliar person. On the basis of the literature and the findings of project No. Z01 HD 00006 three factors were hypothesized to be related to the amount of distress an infant shows to the approach of a stranger: 1) social exposure to only a few adults in early infancy, 2) a temperamental characteristic of high general irritability and 3) a tendency to become upset in day to day separation experiences. The second and third factors proved to be significantly related to the likelihood of showing stranger distress.

Although a history of exposure to few adults in infancy was not significant by itself, this variable appears to have some influence in conjunction with the other variables. This is shown by the relationship between the number of these temperamental and experimental factors in the child's history and the likelihood of showing stranger distress. Nine percent of the infants were upset by the stranger when none of these factors was present; 54% when one of the factors was present; 92% with two of the factors, and 100% when all three factors were present.

Significance to Biomedical Research and the Program of the Institute: The study of "attachment" is central to understanding the development of affectional relationships. Most of the research on attachment has been carried out in contrived situations of a rather stressful nature. There has been little comparison of reactions of infants in these experimental situations with behavior in natural settings.

Proposed Course of Project: Preparation of reports for publication will continue throughout the next fiscal year. Two pilot studies are currently under way into various aspects of attachment. In one study infants are observed in a playgroup under two conditions: with their mothers present and with their mothers absent. In the other study various approaches will be tried to mitigate distress in children who are particularly upset by even minimal separations from parents (such as mother leaving the room). These approaches will be based on different theories of attachment in order to see which of the theories provides the most effective approach to decreasing separation distress.

Publications:

Klein, R. P., & Durfee, J. T. Infants reactions to strangers vs. mothers in R. J. Harmon & G. A. Morgan (Eds.) Infants reactions to unfamiliar adults: A discussion of some important issues. Educational Resources Information Center, ED 115 384, 1976.

Morgan, G. A., & Bennett, C. A. Implications of various methods of scoring infants' reactions to unfamiliar adults. In R. J. Harmon & G. A. Morgan (Eds.) Infants reactions to unfamiliar adults: A discussion of some important issues. Educational Resources Information Center, ED 115 384, 1976.

Morgan, G. A., Levin, B., & Harmon, R. J. Determinants of individual differences in infants' reactions to unfamiliar adults. JSAS Catalog of Selected Documents in Psychology, 1975, 5, 277. (Ms. No. 1006, 19 pages)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00007-02 SBS

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Environmental Influences on Cognitive Development in 13 Month Infants

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Gaiter	Staff Fellow	SBS	NICHD
	L. J. Yarrow	Chief, Social & Behavioral Sci. Br.	SBS	NICHD
OTHER:	B. C. Marcus	Research Psychologist	SBS	NICHD

COOPERATING UNITS (if any)

Parent and Child

LAB/BRANCH
Social and Behavioral Sciences Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.50	.40	.10

SUMMARY OF WORK (200 words or less - underline keywords)

To assess the cognitive content of the play experiences of 13-month-old infants with their parents, siblings and others, and to examine relationships between these experiences and infant cognitive performance.

PHS-6040
(12-75)

Project Description:

Objectives: To assess the cognitive content of the play experiences of 13-month-old infants with their parents, siblings and others, and to examine relationships between these experiences and infant cognitive performance.

Methods Employed: The subjects were 40 full term 13-month-old infants who are part of a study of early cognitive and motivational development (Z01 HD 00008). Data were obtained in a structured session in a laboratory playroom in which there were a variety of tasks designed to provide opportunities for problem-solving and for various kinds of feedback and for practice of emerging skills. The Bayley Scales of Infant Development and the (Uzgiris and Hunt) Object Permanence Scale were also administered in the infants' homes. In addition, mothers were interviewed regarding the kinds of play experiences their infant engaged in routinely with her, her husband and with other familiar people. Mothers reported a variety of cognitive activities such as labelling objects, working with simple formboards and puzzles requiring form discrimination, pretending to be storybook characters, and playing the piano with the baby. The key score derived from these data was the varitty of cognitive activities in play.

Major Findings: The measure of cognitive experience in play in the home, variety of cognitive activities, was found to relate significantly to two motivational measures, a measure of the infant's tendency to practice a developing skill, r=.35, and a measure of his persistence in attempts to solve difficult problems, r= .32. In addition, the variety measure significantly related to the Bayley Mental Developmental Index, r=.45, as well as to two of the four differentiated measures of infant functioning derived from the Bayley. Language, r=.52, and Perceptual Discrimination, r=.39. These findings are consistent with other studies which have shown that a variety of stimulation in everyday home experiences is significantly related to cognitive development in children during the sensorimotor period.

Significance to Biomedical Research and the Program of the Institute: This project adds a significant dimension to the ongoing study of cognitive and motivational development by sampling the stimulation infants receive through play with other people in their home environments.

Proposed Course of Project: All data collection has been completed. The findings will be reported at the regional meetings of the Society for Research in Child Development. Following the meetings these findings will be prepared for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00008-03 SBS

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Measurement of Effectance Motivation in One Year Old Infants

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. J. Yarrow	Chief, Social & Behavioral Sci. Br.	SBS NICHD
	G. A. Morgan	Research Psychologist	SBS NICHD
OTHER:	K. D. Jennings	Staff Fellow	SBS NICHD
	R. J. Harmon	Research Associate	SBS NICHD
	J. L. Gaiter	Staff Fellow	SBS NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Social and Behavioral Sciences Branch

SECTION

Early Development

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.20	2.95	.25

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to clarify the concept of the motivation to master the inanimate environment. Each of the 44 one year old infants was observed in three independent situations designed to measure: free play, cognitive functioning, and mastery motivation. In the mastery motivation session, tasks were developed to elicit: attempts to solve difficult problems, the practice of emerging skills, and the production of effects (feedback). We found high consistency between measures of persistence on conceptually similar tasks administered in different settings, but only modest interrelations among persistence measures on the three different types of mastery tasks. Persistence on the mastery motivation tasks was related to performance on the Bayley Scales of Infant Development, suggesting that infants who show considerable sustained, task directedness develop the cognitive and fine motor skills tested by the Bayley earlier than less persistent infants. Additional support for this suggestion comes from the free play session. There were significant relationships between the amount of spontaneous, cognitively mature play (for example, practicing the emerging skill of combining objects) and performance on the Bayley test and the mastery tasks.

Project Description:

Objectives: Until very recently, much of the research on infant development has been focused on cognitive functioning, with little empirical study of the motivational underpinnings of cognitive development. Although there has been increasing theoretical attention to the motives underlying the development of competence, it has been difficult to operationalize and to measure behaviors which index these motives. A major objective of this research was to develop measures of the infant's active striving to perfect emerging skills, to solve difficult problems, and to act upon the environment to produce effects. We are interested in the beginnings of mastery motivation. We also plan to explore the environmental antecedents of mastery motivation by looking at the relations between the infant's early experiences and mastery motivation at one year.

Methods Employed: The sample consisted of 44 one year old infants from middle-class families, 23 boys and 21 girls. Each infant was observed in three different situations: during free play in our laboratory, during a structured session designed to measure attempts at mastery, and during developmental tests given in their homes. Each of the three sessions was conducted independently; i.e., the infant was seen by a different experimenter observer who had no knowledge of his performance in the other sessions. At 6 months 25 of these babies had been observed in their homes with their mothers and also tested as part of project Z01 HD 00003.

In the free play session, the infant was provided with a wide variety of toys and household objects, while the mother was in the same room being interviewed. A fifteen minute segment from the middle of the play session was videotaped and later coded. The basic data consisted of counts of the number of 20-second intervals in which specific types of behaviors occurred. We were interested in a variety of behaviors. We distinguished exploratory behavior from more cognitively mature play, e.g., combining objects, appropriate use of objects, producing effects. We also coded the infant's sustained attention to a single object, his persistence at difficult tasks and his attempts to solve problems.

In the session developed to measure motivation to master the inanimate environment, there were three types of tasks. The first provided opportunities for the infant to obtain clear visual and/or auditory feedback through his actions, e.g., pulling a lever produced a click and moving lights. The second type of task involved problem solving, e.g., reaching around a glass barrier to obtain a toy. In the third type of task, the infant was given problems which required the use of emerging spatial relations and fine motor skills, e.g., pegs were to be put in holes, and blocks in a bottle. For the three types of task, we derived several measures: 1) a persistence score, a measure of the amount of time he engaged in sustained task-oriented behavior; 2) some measures of the infant's attitude toward the task, such as time before involvement in the task, expressions of positive and negative affect; 3) a measure of his interest in exploration, the amount of time spent exploring the task materials; and 4) a measure of skill, the number of tasks he solved.

In a separate session at 13 months, the infants were given the Bayley Scales of Infant Development and the Uzgiris-Hunt Object Permanence Scale. The Bayley Scales yield indexes of psychomotor development (PDI) and mental development (MDI). To obtain measures of more differentiated aspects of infant functioning, we derived four clusters: Problem Solving, Perceptual Discrimination, Language, and Practicing Spatial Relations Skills which included items requiring repetition of a skill such as putting cubes in a cup.

Major Findings: We first looked at consistency across sessions for similar measures. We found fairly high consistency when the tasks were similar, but similar measures on very different tasks were not highly related. For example, the persistence scores on the three mastery motivation tasks were only modestly interrelated, indicating that these three types of problems tap different aspects of cognitive-motivational functioning. On the other hand, the persistence cluster on the Bayley Examination (Practicing Spatial Relations Skills) was highly correlated (r=.66) with the persistence score on conceptually similar tasks during the mastery motivation session.

Infants who had not yet acquired the cognitive or fine motor skills required to solve a task did not persist at it as much as infants who had these skills, at least in emerging form. Infants who had not completely mastered these skills persisted on those tasks which represented a challenge, tasks which were neither too easy nor too difficult for them. Furthermore, persistence in mastery motivation tasks was significantly correlated with performance on the Bayley Examination (r=.60 with MDI). Similarly, those who persisted on the Bayley Test solved more of the mastery tasks (r=.45). Cross-session correlations were especially high between similar tasks. For example, persistent practicing of spatial relations skills in the mastery motivation session was significantly related to performance on the conceptually similar Bayley Cluster, Perceptual Discrimination (r=.74).

The findings from the free play session suggest that we need to go beyond the simple assumption of a link between the amount of general exploration and cognitive ability. The amount of time the infant spends in exploration shows low and inconsistent relationships with cognitive ability. However, the quality of exploration shows clear relationships to cognitive development. Significant relationships were found between the quality of exploratory play and cognitive performance in the other two sessions, i.e., to the number of problems solved in the mastery session and to the Bayley MDI. Touching, mouthing, and banging probably extract only a minimal amount of information regarding the properties of objects. Behaviors more specifically adapted to the particular object (e.g., combining objects) elicit a greater amount of information and feedback.

On the whole, these results show the intertwining of motivation to master the inanimate environment and cognitive functioning. We find that persistence is not only essential for solution of problems; it is also important in perfecting skills. This interpretation is consistent with Robert White's theorizing which links a sense of competence to effectance motivation.

Significance to Biomedical Research and the Program of the Institute: In recent years the measurement of cognitive development in infancy and early childhood has occupied the center of interest, with the infant's motivation to act on the environment--to master difficult tasks and to produce effects--being essentially ignored. Through their very early visual and manipulative exploration, children learn about the properties of their environment. They also learn what can be done with objects and what effects they can have on them, e.g., move them in space, change their shape, elicit sounds or interesting visual displays. Similarly, in interactions with people, infants learn that if they vocalize or cry, a caregiver is likely to respond to them in some way. These activities with people and objects come to be associated with the infant's sense of competence and feelings that he can control and master his environment. His motivation to produce effects probably is strengthened in these encounters with people and objects. In young infants, effectance production is closely intertwined with cognitive development. The quality of his object manipulations is dependent on his skill and, in turn, influences his acquisition of knowledge and skills. The poor predictability of measures of intellectual development during infancy may be partly related to the failure to consider the role of motivational factors.

Proposed Course of the Project: Data collection and some analyses have been completed. Preliminary results have been presented in a symposium. We plan to analyze the relationships between the infant's early environment at 6 months and these motivational measures at one year. We also plan to look at early precursors of these motivational functions to determine whether there are any relations between neonatal characteristics and infant measures at 6 months and these measures at one year. Next year some of these infants will be studied as preschoolers to determine whether effectance motivation at 12 months is related to later competence.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00009-03 SBS

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Early Mother-Infant Interaction and Development Among Premature Infants

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F. A. Pedersen	Research Psychologist	SBS	NICHD
OTHER:	B. J. Anderson	Staff Fellow	SBS	NICHD
	R. J. Harmon	Research Associate	SBS	NICHD
	R. L. Cain	Research Assistant	SBS	NICHD

COOPERATING UNITS (if any)

Parent and Child, Childbirth Education Associates

LAB/BRANCH
Social and Behavioral Sciences Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: .20	PROFESSIONAL: .15	OTHER: .05

SUMMARY OF WORK (200 words or less - underline keywords)

The basic problem is to determine the extent to which the premature infant's isolation and separation from parents (a consequence of being kept in the nursery) influence the mother-infant interaction and affect subsequent cognitive development. In this study comparisons will be made between two groups of prematures that differ in length of the stay in the isolation nursery but are at low risk of neurological damage. The following areas will be assessed:

1. The quality of the mother's interaction with her infant.
2. The mother's perception of the infant's temperament and early personality.
3. The infant's early cognitive and cognitive-motivational development.

Project Description:

Objectives: The basic problem is to determine the extent to which the premature infant's isolation and separation from parents (a consequence of being kept in the nursery) influence the mother-infant interaction and affect subsequent cognitive development. In this study comparisons will be made between two groups of prematures that differ in length of stay in the isolation nursery but are at low risk of neurological damage. The following areas will be assessed:

1. The quality of the mother's interaction with her infant.
2. The mother's perception of the infant's temperament and early personality.
3. The infant's early cognitive and cognitive-motivational development.

Methods Employed: The sample will consist of 30 premature infants who are judged to be at low risk of neurological damage, 15 of whom had a relatively brief nursery stay (7-10 days) and 15 infants with a longer nursery stay (3-4 weeks). The groups will be matched on the following variables: birth order, socioeconomic level, race, maternal age, sex and age from conception at time of data collection.

There are three phases of data collection that will be concentrated around the 5-month-old period:

1. Mother interview. The mother will complete a Q-sort inventory to characterize the infant's temperament and early personality characteristics.

2. Mother-infant observation. There will be two home observations lasting approximately one hour, each utilizing categories and techniques developed in the Social and Behavioral Sciences Branch for studying the early mother-infant relationship.

3. The infant's early development will be assessed with the Bayley Scales of Infant Development.

Major Findings: Pilot work on all of the assessment procedures has been completed. Attempts to recruit infants meeting the strict medical and social criteria necessary to make this project meaningful have proved very difficult. During this fiscal year there was not sufficient staff time available to work with local pediatric units to recruit suitable participants and carry out the study.

Significance to Biomedical Research and the Program of the Institute: In addition to the possibility of neurological damage, there are several potentially harmful environmental circumstances associated with prematurity. There has been speculation that the early separation from the mother in the nursery adversely affects her capacity to relate to the infant. Isolation of the premature in the nursery is also associated with significant restriction in sensory input, which might have negative effects. This investigation will contribute information on these questions which have implications for the care of premature infants.

Proposed Course of Project: At present we are exploring a collaborative relationship with the Neonatal and Pediatric Medicine Branch, NICHD, in order to carry out this study. We anticipate that this project will undergo some reformulation with new staff involvement.

NICHD Annual Report
Behavioral Biology Branch
July 1, 1975 through June 30, 1976

I. Section on Neurobiology

The cellular and molecular basis for synapse formation and long term modulation of synaptic function are major areas of interest in the Section. A major advance in approaching these problems was made this year in demonstrating that neurons from continuous cell lines were capable of forming synapses with skeletal muscle. We have shown that these synapses are cholinergic and have many of the characteristics of immature synapses *in vivo*. The hybrid cells we have been studying have been shown by us and by others to possess a number of types of receptors of neurobiologic interest and show electrical responses to dopamine, morphine, acetylcholine, prostaglandin and serotonin. We have begun to work out the mechanisms of these receptor mediated responses and to study their relationships to synaptic action.

Pharmacologic studies of chemical and electrical excitability in normal nerve cell cultures have continued. A prototypic anesthetic agent, pentobarbital, has been shown to have selective effects on neuronal responses elicited by glutamate, an excitatory agent and γ-aminobutyric acid, an inhibitory agent. Glutamate elicited depolarization of the neuronal membrane is decreased or abolished while GABA elicited hyperpolarization is prolonged and converted into a slight depolarization.

Synaptic or action potential activity may be coupled to the metabolic activity of neurons and mechanisms which may be related to such coupling are of substantial interest. Different nerve cells utilize different ionic mechanisms in producing active potentials; dorsal root ganglion cells develop Ca^{++} action potentials in part while spinal cord cells have action potentials solely dependent on Na^{+}. The endoplasmic reticulum of neurons appears to serve as a Ca^{++} sequestering site and sub-surface cisterns in some nerve cells may serve as a means by which surface membrane events (synaptic or action potentials) may couple into this intracellular membrane-bounded system.

Work has continued on an inductive interaction between muscle cells and spinal cord cells in culture. Conditioned medium (CM) from muscle cultures induces an increase in the choline acetyltransferase (CAT) activity of spinal cord (SC) cells. The inductive activity is due to material of > 50,000 daltons. Further characterization of the material and its mechanism of action are needed.

It appears that transcriptional diversity in neural tissue is markedly affected by a number of experimental procedures. Total tissue RNA is hybridized with unique DNA sequences as the index of this diversity. Rats raised in an enriched environment exhibit 20-30% greater brain RNA diversity than do rats raised in an impoverished environment. No change is found in liver RNA diversity. An experiment allowing greater experimental manipulation can be done with controlled differentiation of neuronal cell lines. Treatment of a neuroblastoma clone with bromo deoxyuridine produces morphologic and electrophysiologic differentiation and this is accompanied by a significant increase in RNA diversity. These behavioral and tissue culture models represent favorable material for the analysis of the regulation of gene action in the nervous system.

II. Section on Functional Neurochemistry

The major focus of the section is to study peptides and proteins which are involved in intercellular communication (i.e., as neurotransmitters, modulators, and 'trophic' agents). Because of the intrinsic heterogeneity of nervous tissue, a significant effort has been made in the area of microanalytical technology, so that individual neurons and small regions of brain may be studied biochemically as well as electrophysiologically.

The identifiable neurons from the ganglia of molluscs, Aplysia and Otala, have served as excellent model systems for these studies. In the Aplysia, neuron R_2 represents a prototype cholinergic neuron, whereas R_{14}, R_{15}, and the bag cells are typical peptidergic neurosecretory cells. R_{15} contains a peptide which acts as an anti-diuretic hormone, while the bag cells' peptide induces egg-laying in the animal. The biosynthesis, axonal transport, and regulation of these peptides in these neurons have been intensively studied in this section. As a result of this work several generalizations have been made: 1) the peptide hormones are first synthesized as protein precursors which then undergo post-translational cleavage; 2) this processing of the precursor appears to occur in the Golgi as well as in the neurosecretory granules (evidence from subcellular fractionation and autoradiography); 3) processing can occur intragranularly during axonal transport; 4) the rate of clearance of newly synthesized peptides is directly correlated with spike frequency (i.e., $R_{15} > L_{2-6} > R_{14}$), and 5) synaptic (dopamine) or behavioral input to the neuron can regulate synthesis and transport of the peptide. This work has led to a model which is continually being tested, and attempts to relate this model to vertebrate systems are in progress (see below).

The neurohypophyseal peptides, oxytocin and vasopressin, have long been assumed to follow the biosynthetic and transport cycle described above. Our recent experiments on rats have directly demonstrated that these peptides are synthesized in the hypothalamus as protein precursors and then converted during axonal transport to the peptide hormones and neurophysin carrier proteins. Several other peptides are also synthesized, para passu, and are found in the pituitary. These peptides heretofore unreported may be significant for pituitary function. We have shown that synthesis of the precursor is increased 5-10 fold by osmotic stress of the animal, thus providing an excellent model for peptide regulation.

The amphibian pars intermedia is another peptide releasing system uniquely suited for regulatory studies. In the toad the release of MSH is regulated by inhibitory control via dopamine, whereas in the chameleon the control is excitatory through serotonin. Comparison of the biosynthetic and release mechanisms in these two species (in combination with behavioral experiments) are currently underway in the section, and should provide insight into the control of release of peptides. In this and in the above studies, electrophysiological and pharmacological experiments are performed as well.

Attempts are being made to localize peptide hormones (e.g., gastrin, ACTH, glucagon, etc.) in various brain regions using radioimmunoassay techniques. In addition, studies on peptide synthesis and release in hybrid neuroblastoma cells have been initiated in collaboration with the Section on Neurobiology in this Branch.

Experiments done at the Marine Biological Laboratory, Woods Hole, Mass., on the giant axon of the squid have shown that large proteins (> 200,000 daltons) are synthesized by Schwann cells and transferred into the giant axon. To our knowledge this is the first unequivocal demonstration of macromolecular transfer between glia and neurons, in situ. This work led to our demonstration that in crayfish axons the transmitter enzyme for GABA, glutamic acid decarboxylase, is synthesized in the glia surrounding the GABA containing axons. In the crayfish axons, the surrounding glia appear to be essential for maintenance of axonal function.

Recent studies, conducted at the Marine Biological Laboratory in conjunction with Dr. I. Tasaki's laboratory, on the squid giant axon have shown that certain proteins on the inside surface of the plasma membrane are released by depolarization. This release is dependent upon the presence of calcium fluxes during depolarization. The excitability of the axon was inversely related to the amount of protein released from the membrane. Thus, a significant degree of regulation of excitability may be associated with loosely bound proteins on the internal surface of the membrane.

III. Section on Brain and Behavior

Neurophysiological studies of central auditory mechanisms in the squirrel monkey utilizing species typical vocalizations as stimuli have continued during the past year. An understanding of the contribution of several relevant brain regions to neural detection and encoding in this animal communication system is sought by painstaking study of samples of single cell responses in the brains of awake monkeys.

Two substantial bodies of data have been collected during FY 1976. First, Dr. Newman (on leave during all of FY 1975 and 4 months of FY 1976) completed an examination of 300 cells in the dorsolateral frontal cortex. This work was conducted in collaboration with Dr. David Lindsley at the Max Planck Institut, Munich, Federal Republic of Germany. A relatively low percentage of cells (30%) were found which responded to vocal stimuli, and the nature and timing of such responses suggest that this area of the brain does not contribute to processing of vocal stimuli at a level higher than that found in the temporal auditory areas. That auditory processing which is found appears to include a contribution of frontal cortex to selective detection of noisy vocalizations. Noisy vocalizations are regularly employed by squirrel monkeys in agonistic interactions and a link between the auditory processing system and limbic centers is therefore implied.

The second area of neurophysiological study which has been completed during the year involves examination of the characteristics of cortical regions in the temporal lobe which surround the primary auditory projection; the so-called "secondary" areas. A sample of 175 cells within the superior temporal gyrus and situated at varying distances from the primary projection has been studied. The data are still being analysed, but it is clear that the secondary areas have lower overall responsiveness (about 65% of cells can be driven by vocalizations as opposed to 90% in primary cortex) and contain more cells which are highly selective to one or a few vocalizations. It has not been possible so far to identify a particular subregion as containing a concentration of such "language specialists" and thus qualify as a precursor of the language areas of the human brain. It should also be noted that these and previous studies have not disclosed systematic differences between left and right hemispheres.

Investigations expanding the above have been started. An additional cortical area with known auditory inputs (post-arcuate frontal cortex, located just posterior to previously studied frontal cortex) is under study, and examination of cells in the thalamic relay nuclei of the auditory system has also begun in late FY 76.

Additionally, anatomical studies have been initiated utilizing double labeling techniques with horseradish peroxidase and radioactive amino acid injections. The purpose of such studies is to develop a body of evidence presently unavailable in the squirrel monkey regarding the destinations and probable sites of information transfer of the cortical auditory projections.

Studies on vocal behavior and its development in squirrel monkeys have continued, but new initiatives in behavioral genetics have run into difficulties. Health problems in the animal colony severely restricted our breeding program, and a planned study on mother-infant vocal recognition could not be carried out due to insufficient subjects. Our longitudinal examination of dialect development has moved forward, and 8 laboratory reared infants have now been characterized vocally. Adequate data on both parents exists for only 5 infants, but it is abundantly clear that vocal characteristics of both mother and father are found with the former dominant. Plans have been made to get the breeding program back on schedule utilizing in-house facilities and improved health screening of breeding stock and better isolation of breeding groups from laboratory disturbances.

A fruitful collaboration was begun during FY 1976 with the Section on Neuropsychology, NIMH, involving developmental studies on frontal lobe function in juvenile Rhesus monkeys. The technique of brain cooling has been applied to the problem of maturation of dorso-lateral frontal cortex, allowing repeated assessment of residual function in the same animal. Results of this study demonstrate that removal by cooling of frontal cortex does not impair delayed response performance in monkeys under 2 years of age but has a disrupting effect on this behavior at 2 years. Since the behavioral tasks can be performed perfectly by the younger animals, the hypothesis is generated by these findings that related subcortical structures may be functional when cortex is immature. This hypothesis is being tested by Dr. Alexander in collaboration with Dr. Goldman of NIMH by combining cooling and single cell recording in performing monkeys.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00046-07 BB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Physiologic studies of neurons and other cell types in cell culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. G. Nelson	Chief, Behav. Biol. Branch	BB	NICHD
	B. R. Ransom	Medical Officer	BB	NICHD
	C. N. Christian	Guest Worker	BB	NICHD
OTHER:	R. W. Holz	Guest Worker	BB	NICHD

COOPERATING UNITS (if any)

Section on Technical Development, NINCDS-NIMH
Laboratory of Biochemical Genetics, NHLI

LAB/BRANCH

Behavioral Biology Branch

SECTION

Section on Neurobiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.8	3.8	4.0

SUMMARY OF WORK (200 words or less - underline keywords)

A continuous hybrid cell line, formed from a mouse neuroblastoma and a rat glioma has been shown to form functional synapses with normal mouse myotubes in culture. These cells can be maintained in continuous logarithmic growth or can be caused to differentiate by treatment with dibutyryl cyclic adenosine monophosphate (db cAMP) or X-irradiation. The cells are electrically responsive to serotonin and acetylcholine. Receptors for a number of neuroactive compounds including narcotics have been demonstrated by others. They represent an attractive model system for structure of synapse development and modulation. Fundamentally different mechanisms are involved in the action potentials (A.P.) generalized by different types of mouse neurons in culture. Dorsal root ganglio cells exhibit both Ca^{++} dependent and Na^{+} dependent components; this latter component is largely tetrodotoxin (TTX) insensitive. Spinal cord neurons by contrast, are exclusively due to Na currents and these are completely TTX sensitive.

PHS-6040
(12-75)

Project Description:

Objectives: To understand the cellular molecular mechanisms relevant to specificity of synapse formation and factors involved in the modulation of the strength of synaptic connections.

Methods Employed: A number of methods for maintaining and differentiating the cell lines have been devised in the Behavioral Biology Branch and in the Laboratory of Biochemical Genetics, NHLI. Electrophysiologic intracellular microelectrode techniques have been used for studying electrical excitability, chemosensitivity and intercellular communication in the cultured cells. An optimal culture regimen has been developed for producing well-differentiated mouse myotubes.

Major Findings: When cells of the NG108-15 hybrid cell line (neuroblastoma x glioma somatic cell hybrid) are grown in db cAMP their mitosis rate markedly slows and they undergo considerable morphologic differentiation. It had been shown by others that this cell line expressed choline acetyltransferase and, with db cAMP treatment, synthesized stored and probably secreted acetylcholine. When we plated these differentiated hybrid cells on optimally developed normal myotubes and maintained these co-cultures for periods of several days or more functional synapses developed between the hybrids and the muscle cells. The synapses were demonstrated by recording intracellularly from hybrid-muscle pairs, stimulating the hybrid and observing synaptic potentials in the muscle cells. These synapses are cholinergic (since they are blocked by d-tubocurarine and α-bungarotoxin), require calcium in the extracellular fluid and operate by a conventional postsynaptic conductance change mechanism. The nature of the transmission is similar in many ways to developmentally very early stages of neuromuscular synapses in vivo.

The hybrid cells are sensitive to serotonin, dopamine and acetylcholine and respond to iontophoretic application of these agents with large depolarizations of the cell membrane potential. Focal application of serotonin to contact sites between the hybrid and muscle cells results in release of transmitter from the hybrid and production of synaptic potentials in the muscle cells. Serotonin further appears to increase the size of muscle synaptic potentials evoked by action potentials in hybrid cells innervating the muscle.

Work has continued on investigating normal spinal cord and brain cells in culture under this project. Glycine has been compared with gamma aminobutyric acid (GABA) with regard to chronic effects on inhibitory synaptogenesis. Glycine was found to be much more potent in this regard than GABA in spinal cord cultures. Spinal cord (SC) cells have been shown to develop action potentials (A.P.) which are due exclusively to tetrodotoxin sensitive sodium currents, while the A.P. of dorsal root ganglion cells are due to both Ca^{++} and Na^+ currents. The DRG A.P. Na^+ dependent A.P. component is resistant to tetrodotoxin.

Significance to Biomedical Research and the Program of the Institute: The clonal neuroblastoma system has been used in a large number of studies related to a variety of neurobiologic properties (e.g., neurite formation, receptor synthesis, transmitter related enzyme regulation). Heretofore, this system has been a markedly deficient system in that the capacity for synapse formation had not been demonstrated. The fact that conditions can be found in which these cells do form synapses allows this clonal system to be exploited for this most interesting neurobiological problem of synaptogenesis. The findings indicate that the initial stage of synapse formation need not involve a high degree of specificity and that a number of probably non-genetic selective mechanisms are presumably involved in highly organized synaptic connections.

Proposed Course: Possible specificity in synaptogenesis needs to be explored with a variety of neuroblastoma and hybrid clones. Biochemical assays for transmitter release from the hybrid cells are being developed so this important process can be efficiently studied. A number of factors will be assayed for their effects on the efficacy of synapses (chronic electrical stimulation, cyclic nucleotide treatment, prostaglandins, neurohormones).

Publications:

1. Nelson, P. G.: Central nervous system synapses in cell culture. In Cold Spring Harbor Symposium on Quantitative Biology, Vol. 40, 359-371, 1976.

2. Nelson, P. G. and Christian, C. N.: Neuronal cell cultures and their application to the problems of plasticity. In Rozenzweig, M. R. and Bennett, E. L. (Eds.): Neural Mechanisms of Learning and Memory. Cambridge, MIT Press, 1976.

3. Nelson, P., Christian, C. and Nirenberg, M.: Synapse formation between clonal neuroblastoma x glioma hybrid cells and striated muscle cells. Proc. Nat. Acad. Sci. U.S. 73: 123-127, 1976.

4. Ransom, B. R., Barker, J. L. and Nelson, P. G.: Two mechanisms for post-stimulus hyperpolarizations in cultured mammalian neurons. Nature 256: 424-425, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00047-07-BB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biochemical studies of neuronal and other cell types in cell culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. K. Schrier	Medical Officer	BB	NICHD
	P. G. Nelson	Chief, Behav. Biol. Branch	BB	NICHD
	Y. Sarne	Visiting Fellow	BB	NICHD
OTHER:	J. H. Neale	Guest Worker	BB	NICHD
	E. W. Godfrey	Biol. Laboratory Technician	BB	NICHD
	M. M. Mata	Visiting Fellow	BB	NICHD
	E. A. Neale	Staff Fellow	BB	NICHD

COOPERATING UNITS (if any)

C. Y. Chiou, Laboratory of Biomedical Sciences, NICHD
D. C. Klein, Laboratory of Biomedical Sciences, NICHD

LAB/BRANCH

Behavioral Biology Branch

SECTION

Section on Neurobiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	1.7	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

Cultured cell systems are being used to investigate aspects of nervous system development and regulation of function. Specifically, (1) the induction, mediated by muscle conditioned medium, of choline acetyltransferase activity in spinal cord cell cultures was found to require a high molecular weight factor or factors which could also be obtained from an homogenate of embryonic muscle. (2) Cerebellum cells from newborn rats were found to adhere rapidly and specifically to a coating of polylysine on culture dishes; this adhesion can be modified by various treatments to cell membranes, such that an investigation of the surface moieties responsible for the adhesion is being performed. (3) Cultures prepared from cerebellum cells of 16 day fetal rats were found to maintain their large neurons for long periods without high [K^+] in the culture medium. Such cultures are being employed for developmental studies of the electrical and biochemical properties of the cells. (4) Cultures of pineal cells were established and studied extensively for morphologic and biochemical characteristics. These cultures mimic normal pineal glands in most regards, but differ in some aspects of morphology and biochemistry.

Project Description:

Objectives: (1) Evaluation of the trophic effects of muscle cells and muscle culture conditioned medium on spinal cord cells, specifically to determine the nature of regulation of choline acetyltransferase activity in spinal cord cells. (2) Continued use of cell culture methods to investigate the functions of neurons, glia, and other cells from the nervous system.

Methods Employed: Most of the techniques for cell culture, and biochemical and morphologic evaluation have been described in previous reports. In addition, we have developed methods for assay of binding of cerebellum cells to polylysine-coated dishes and for obtaining and culturing reproducibly cells from pineal glands and superior cervical ganglia of rats.

Major Findings: a) Attempts were made to purify the nervous system enzyme choline acetyltransferase in order to obtain antibodies to it, and also to employ an antibody preparation made against impure choline acetyltransferase to determine the rates of synthesis and degradation of the enzyme before and during induction of its activity by co-culture with muscle cells; these attempts had to be abandoned. Our efforts subsequently have been concentrated on determining the nature and action of the inducing factor (or factors) in muscle conditioned medium. The factor is > 50,000 molecular weight and can also be found in homogenates of skeletal muscle tissue from 15-20 day embryonic mice.

b) Although the enzyme glutamate decarboxylase (GAD) and its neuroactive product GABA were found in pineal glands, we found that the enzyme was not associated with nerve endings within the gland from cell bodies in extra-pineal locations. GAD activity was not regulated by any known endogenous rhythm or neural input to the gland. In addition, we were unable to demonstrate any effects of GABA either pre-or post-synaptically on the cyclic AMP-mediated catecholamine-stimulated induction of serotonin N-acetyltransferase activity in the gland.

c) Cells dissociated from cerebella of young rats became adherent to coatings of polylysine on polystyrene dishes within 20 hours of inoculation; this was much more rapid than adhesion to either a coating of collagen or polystyrene alone. The affinity for polylysine was greatest at 1-2 days of age and decreased for cells from cerebella of younger and older animals. Cells from cerebral cortex had no greater affinity for polylysine than for the other surfaces. Binding of cerebellar cells was found to be ionic in nature, as negatively charged particles competed with the cells for binding; binding was inhibited by treatment of the cells with neuraminidase and trypsin. but not by hyaluromidase, Ca^{++} ions or by the Ca^{++} chelator EGTA. The binding site on the cells may be a neuraminic acid moiety.

d) Cultures of cerebellum cells from 16 day embryonic rats contain some large neurons which survive for long periods of time. In contrast to cells from newborn or older animals, cultures of these cells do not require high concentrations of K^+ ions (24 mM) in the medium in order to maintain the neurons.

These cultures are being employed for studies of the electrophysiologic responses of the neurons to applied neuroactive substances and of the development of GAD activity.

e) Cultures of pineal cells were found to contain both "light" and "dark" pinealocytes, some of which contained cilia, vesicle-crowned rodlets (ribbon synapses), and subsurface cisterns; muscle cells, fibroblasts, and cells considered to be astrocytes. The cultures responded to treatment with norepinephrine, dibutyryl-cyclic AMP and dibutyryl-cyclic GMP alike with several-fold increases in NAT activity. The effect of norepinephrine could be almost completely blocked by prior treatment with either β_1 or β_2 adrenergic blocking agents. Half maximal response was obtained with 7.4×10^{-9}M norepinephrine. The cultures were characterized by a very high basal activity of NAT, as if stimulation (or the absence of repression) occurred continously during culture. The induced level of NAT activity was similar to that obtained under maximal stimulation in organ culture. The pineal cell cultures supported the development and differentiation of neurons from rat superior cervical ganglia; an attempt to identify nerve growth factor in the cells or medium was unsuccessful.

Significance to Biomedical Research and the Program of the Institute: The regulation of neurobiologic functions is very important to development of the entire organism, and many of these regulatory steps may, in fact, determine the course of embryogenesis. a) The study of trophic interactions between muscle and spinal cord, an apparently normal phenomenon which is reproduced in cell culture, provides a system in which abnormal myoneural interactions, such as those in some neuromuscular diseases, may be evaluated. b) The nature of the external cerebellar cell membrane at certain times in development may be of importance in understanding mutations in systems which control cell migration and development. c) The pineal gland is important in gonadal function as well as in the mediation of circadian rhythms; studies on the presence and function of GAD and GABA in the pineal may provide insight into the relationship between glia and neurons. d) The pineal cell cultures give us the opportunity to dissect pineal morphology, biochemistry and physiology and ultimately perhaps to understand the function and interrelationships of the cell types in the pineal gland.

Proposed Course: We plan to continue to investigate the trophic relationships between muscle and spinal cord cells in culture, to isolate and describe the inducing factor(s) and gain insight into the mechanism of this induction. We plan soon to complete our investigation of moieties on cerebellar cell surfaces which govern adhesion to polysine, although biochemical and electrophysiological characterization of fetal cerebellum cell cultures will continue. Our studies with pineal GAD and GABA are essentially completed; additional investigations of the interactions between pineal cells and sympathetic neurons are planned.

Publications:

1. Miller, L. H., Shiroishi, T., Dvorak, J. A., Durocher, J. R. and Schrier, B. K.: Enzymatic modification of the erythrocyte membrane surface and its effect on malarial neurozoide invasion. J. Molec. Med. 1: 55-63, 1975.

2. Sarne, Y., Schrier, B. K. and Gainer, H.: Evidence for local synthesis of a transmitter enzyme (glutamic acid decarboxylase) in crayfish peripheral nerve. Brain Research. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00048-02 BB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies of transcription level control of neurobiologic and behavioral phenomena

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. D. Grouse	Research Associate	BB	NICHD
	B. K. Schrier	Medical Officer	BB	NICHD
OTHER:	P. G. Nelson	Chief, Behav. Biol. Br.	BB	NICHD
	J. M. Grouse	Guest Worker	BB	NICHD
	D. S. Grouse	Guest Worker	BB	NICHD

COOPERATING UNITS (if any)
Dr. Edward L. Bennett, Laboratory of Chemical Biodynamics, Univ. of California
Dr. Mark R. Rosenzweig, Dept. of Psychology, University of California, Berkeley

LAB/BRANCH
Behavioral Biology Branch

SECTION
Section on Neurobiology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

SUMMARY OF WORK (200 words or less - underline keywords)

Molecular hybridization of cellular RNA with unique sequences of DNA is being employed as a measure of gene expression in several model systems in which modification of nervous system - related functions are manipulable by environmental modification or disease states. In mostly preliminary data several model systems have shown promise of providing easily measurable changes in gene expression (RNA diversity). These include: (1) visual cortices and brain stems of kittens whose eyelids are sutured closed; (2) whole brains of rats raised in enriched vs. impoverished environments; (3) morphological and physiological differences under different growth conditions in cultured cell lines of neural or muscle origin; and (4) muscle and nerve tissue from normal mice and those with hereditary muscular dystrophy. The studies are continuing.

Project Description:

Objectives: In this project we are investigating the extent and control of RNA sequence diversity in several manipulable systems with particular attention to the nervous system and development.

Methods Employed: As described in the previous project report, these studies employ hybridization of cellular RNA in vast molar excess with unique sequences of homologous DNA. The DNA which is made double-stranded in the reaction can be isolated and the extent of reaction is a measure of gene expression (RNA diversity) in the tissue from which the RNA was obtained.

Major Findings: a) In three successive studies with a total of 28 RNA preparations we found consistent and significant elevations in brain RNA diversity in rats raised in enriched environments compared to their littermates raised in impoverished environments. The changes were similar to, but much greater than the changes in brain weights determined by Bennett for these same animals.

b) We looked for differences in RNA diversity in two subclones obtained from a previously cloned rat muscle cell line in which cell morphology had become inhomogeneous. One of these subclones was morphologically epithelioid and did not fuse to form myotubes; the other subclone contained mostly spindle-shaped cells which were capable of fusion into myotubes. Under similar growth conditions there was no difference in RNA diversity between the two clones; 3.1% of the unique sequences in the rat genome were expressed in each.

c) In one subclone of mouse neuroblastoma cells, we found that RNA diversity was markedly diminished, as expected, after treatment with actinomycin D; that treatment with medium without serum, which caused some morphological differentiation, did not significantly change the RNA diversity as compared to controls; and that treatment of the cells with BrdU, which causes similar morphological changes, increased RNA diversity in the cells.

d) In three litters of kittens, some of whose eyelids were sutured closed before natural eye opening, there was a significant decrease in RNA diversity in visual cortices in those sutured animals as compared to littermate controls.

e) RNA samples from liver, spleen, brain, spinal cord and skeletal muscle of normal and "dystrophic" mice have been analyzed for diversity, and marked decreases were found in brain and muscle of dystrophic animals. Additional dystrophic and normal animals, including more exact age-matched pairs, are being collected in order to confirm and extend these findings.

Significance to Biomedical Research and the Program of the Institute: It is our feeling that the ultimate level of control of many experimental, behavioral and species-specific neurobiologic phenomena will prove to be at the level of transcription of the genome. Since Bennett, et. al., have shown differences in some parameters of brain development in rats raised in enriched as compared to impoverished environments, this seemed a good model for study and one that may give information as to the contribution of environment to human brain development. The more simplified systems (e.g., cell cultures) provide the possibility of more clearly dissecting functional control mechanisms by which we may explain nervous system plasticity. Finally, it is hoped that studies of control in disease states may provide insight into the basic mechanisms of those diseases.

Proposed Course: As noted in each of the experimental findings above, none of these studies is complete, but the data clearly show that substantial changes in gene expression result from behavioral or environmental manipulation. It is our plan to concentrate directly on one or two of these experimental models with a view to a eventual clear delineation of the control mechanisms involved.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00049-02 BB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Axoplasmic transport _in_ _vitro_ and _in_ _vivo_

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. H. Neale	Guest Worker	BB	NICHD
OTHER:	E. A. Neale	Staff Fellow	BB	NICHD
	J. L. Barker	Medical Officer	BB	NICHD
	P. G. Nelson	Chief, Behav. Biol. Branch	BB	NICHD

COOPERATING UNITS (if any)
W. Bonner, Reproduction Research Branch, NICHD

LAB/BRANCH
Behavioral Biology Branch

SECTION
Section on Neurobiology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0

SUMMARY OF WORK (200 words or less - underline keywords)

This project has concentrated on the _functional roles_ of proteins which are _rapidly transported_ by axons in the nervous system by first examining the species of rapidly transported proteins in functionally distinct systems (sensory, motor and sympathetic). The results show that a number of distinct classes of proteins heavily labelled with methionine and of the same approximate molecular weights are transported in the functionally different systems. The degree of coincidence of rapidly transported proteins has been further enhanced by separating the methionine-labelled proteins on SDS polyacrylamide slab gels and examining autoradiographs of the separated proteins. These rapidly transported species thus appear to be involved in some processes basic to all nerve cells rather than peculiar to any one functional type of cell. Study of the functions of the rapidly transported species has begun with 1) investigation of Ca^{++} transport in the same systems and search for a rapidly transported protein with high affinity for Ca^{++} and 2) experiments on the distribution of autoradiographic grains in the spinal cord following transport of labelled proteins in the sensory system.

PHS-6040
(12-75)

Project Description:

Objectives: To determine the physiological roles of rapidly transported proteins in the nervous system using in vitro and in vivo systems.

Methods Employed: The peripheral nervous system (sensory and sympathetic ganglia with axons) and spinal cord is isolated from the frog and placed in a chamber appropriate for selective labelling of various parts of the system. The system under study is incubated with radioactive amino acid and allowed to incorporate this into proteins for transport from the cell bodies. The labelled, transported proteins are then homogenized and separated on SDS-polyacrylamide gels, which are either sliced and counted or autoradiographed. Studies on Ca^{++} transport follow the same paradigm except that the $^{45}Ca^{++}$ in various nerve segments is extracted with TCA and then counted. Studies on autoradiographic grain distribution have utilized both excess cold amino acid and protein synthesis inhibitor to block re-utilization of transported label for protein synthesis.

Major Findings: At least eight different molecular weight classes of methionine-labelled proteins appear to be rapidly transported in sensory, motor and sympathetic nervous systems of the frog. These commonly transported species have also been reported in the rabbit visual system and toadfish sonic motor system, suggesting that their functions are basic to the processes of rapid axonal transport. A number of the protein species are also dominate pre-synaptic terminal fractions from rat brain, suggesting that these protei may be destined to play roles at the synapses. $^{45}Ca^{++}$ is also rapidly transported in all the systems studied, including an invertebrate. It may either be part of the rapid transport mechanism or transported intra-organelle, or in association with one or more of the rapidly transported proteins. Preliminary analysis of the distribution of grains in the spinal cord following transport in the sensory afferents shows 1) a much wider distribution of primary afferents than had been previously reported and 2) the appearance of grains in discrete spinal nerve cells. The latter is suggestive evidence for trans-synaptic transport of proteins. A separate series of experiments has been carried out examining the role of Ca^{++} in the synthesis conversion and transport of low molecular weight proteins in molluscan neurosecretory cells. Preliminary analysis show that Ca^{++} is required for transport of the rapidly turning over, low molecular species from the cell body and may be a role in the synthesis of these proteins. Intracellular conversion of higher to lower molecular weight species is unaffected.

Significance to Biomedical Research and the Program of the Institute: A basic understanding of the transport of material within nerve cells and from cell to cell is prerequisite to understanding various pathophysiologies which may affect these processes. At present, our knowledge of these processes which are clearly basic to neuronal function is quite rudimentary.

Proposed Course: Further analysis of data already obtained in the slab gel experiments and in the grain distribution experiments is planned. Also planned are experiments directed at investigating the presence of a rapidly transported protein species with high affinity for Ca^{++} and a study of the release of Ca and transported protein from nerve endings.

Publications:

Barker, J. L., Neale, J. H., and Gainer, H.: Rapidly transported proteins in sensory, motor, and sympathetic nerves. Brain Res. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00050-04 BB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Fine structure and function of the endoplasmic reticulum of neurons

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Henkart	Staff Fellow	BB	NICHD
OTHER:	J. L. Barker	Medical Officer	BB	NICHD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Behavioral Biology Branch

SECTION
Section on Neurobiology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project has been to study the handling of calcium in neurons especially as it involves the endoplasmic reticulum. The approaches taken include: 1) localization of Ca-binding sites; 2) attempts to localize Ca stores; 3) using the freeze-fracture techniques to compare structure of junctions between ER and surface membrane of neurons with similar junctions in muscle; 4) studying the appearance of the ER with various conditions known physiologically to alter the amount of Ca in the cytoplasm; 5) correlation of the amount of surface membrane involved in subsurface cistern junctions and the prominance of Ca current during action potentials in various neuronal types; and 6) correlation of a pattern of distribution of subsurface cisterns with bursting membrane potential behavior.

Project Description:

Objectives: To develop and apply methods for determination of sites of Ca binding or accumulation and to correlate fine structural changes in neurons with physiologically demonstrable effects of Ca entry or release of Ca from internal stores.

Methods Employed: New attempts have been made to localize Ca present in intracellular compartments by treatment of cells with oxalate in the presence or absence of high K^+ solutions or by addition of oxalate with detergents during fixation. The fine structure of nerve cells undergoing slow membrane potential oscillations associated with bursts of action potentials ("bursting") has been compared with the structure of cells not bursting.

Major Findings: In *Aplysia* neurons and cultured mouse or chick spinal cord neurons small amounts of dense precipitate are found in the endoplasmic reticulum after treatment of the cells with oxalate in the presence of high K^+ concentrations. These precipitates may correspond to sites of Ca-localization rather than Ca binding sites. The pattern of distribution and size of subsurface cisterns (SSC) appears to differ in cells which are bursting and cells which are not bursting. In non-bursting cells more of the surface membrane is involved in SSC and the volume of the SSC is greater. Bursting cells have SSC localized primarily in areas of the membrane at the bases of deep glial invaginations opposite areas of restricted extracellular space. These distinctions are apparent in cells that normally do and do not burst as well as in the cells that are made to burst by various manipulations of the ionic composition of the physiological saline.

Significance to Biomedical Research and the Program of the Institute: This study has provided evidence that in nerve cells the endoplasmic reticulum may function as a calcium sequestering system and that surface membrane activity may be coupled to intracellular events via Ca released from the ER. This probably represents an important control mechanism for many cellular functions important in the normal development and maintenance as well as the normal electrical activity of neurons.

Proposed Course: X-ray analysis should be used for identification of the ionic composition of the dense deposits produced by various techniques used in this project. Many new questions have been raised by the results of this project. They will be pursued elsewhere.

Publications:

1. Henkart, M. and Hagiwara, S.: Localization of calcium binding sites associated with the calcium spike in barnacle muscle. *J. Mem. Biol.* In press.

2. Henkart, M., Landis, D. M. D. and Reese, T. S.: Similarity of junctions between plasma membranes and endoplasmic reticulum in muscle and neurons. *J. Cell Biol.* In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00051-03 BB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Changes in membrane properties and fine structure associated with cell morphology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Henkart	Staff Fellow	BB	NICHD

COOPERATING UNITS (if any)

P. A. Henkart, Immunology Branch, National Cancer Institute

LAB/BRANCH

Behavioral Biology Branch

SECTION

Section on Neurobiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project was to elucidate the mechanisms underlying cellular remodeling in different cell types in response to different agents. The response of L-cells to cyclic AMP and changes of shape of Fc receptor bearing lymphocytes in response to immobilized antigen antibody complexes have been studied. Methods employed included time-lapse photography, electron microscopy, treatment with various drugs, and in the case of L-cells, electrophysiology. The roles of various cellular organelles in shape changes have been outlined and some suggestive evidence as to some control mechanisms has been obtained. There are differences between cell types in the detailed patterns of changes involved in the metamorphosis from one shape to another.

Project Description:

Objectives: To elucidate the role of the macrotubule bundle (MTB) in the shape change of Fc-receptor bearing lymphocytes in response to binding of immobilized antigen-antibody complexes.

Methods Employed: Horseradish peroxidase (HRP) - anti-HRP complexes were used to label unseparated human lymphocytes in suspension. They were then fixed and attached in monolayer to poly-l-lysine-coated plastic. Serial sections of the monolayer were analysed to assay the correlation between the Fc-receptor as demonstrated by binding of HRP-anti-HRP complexes and the presence of macrotubule bundles (MTB).

Major Findings: At least 97% of the cells that bear Fc-receptor (FcR) contain macrotubule bundles. All cells that contain MTB were positive for FcR. No cells lacking FcR contained MTB. Since FcR-bearing cells are the only subpopulation of human lymphocytes known to be capable of undergoing a very rapid (within 90 sec) change of shape and onset of motility and since the MTB undergo characteristic changes in morphology in cells that have elongated (as described in last years' annual report) it seems possible that MTB are somehow associated with the shape changes.

Significance to Biomedical Research and the Program of the Institute: The FcR-bearing lymphocyte system seems well suited to study of the coupling between binding of ligands at the membrane and alterations in cellular machinery finally expressed as changes in cell morphology. This is one of the fundamental questions in cell biology. An unforeseen result of this study has been the discovery of an ultrastructural marker for one subpopulation of human peripheral blood lymphocytes.

Proposed Course: We intend to determine whether MTB are composed of tubulin by use of indirect fluorescence labelling with anti-tubulin antibody. Other aspects of this project will be pursued elsewhere.

Publications:

1. Henkart, M. and Henkart, P.: The macrotubule bundle: a cytoplasmic organelle characteristic of normal human blood lymphocytes bearing the Fc receptor. Proc. Nat. Acad. Sci. U.S. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00052-08 BB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biosynthesis of polypeptides in molluscan neurosecretory cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y. P. Loh	Visiting Fellow	BB	NICHD
OTHERS:	H. Gainer	Head, Section on Funct. Neurochem.	BB	NICHD
	Y. Sarne	Visiting Fellow	BB	NICHD
	E. Neale	Staff Fellow	BB	NICHD

COOPERATING UNITS (if any)
M. Daniels, Laboratory of Biochemical Genetics, National Heart and Lung Institute
R. Ruchel, Max Planck Fellow, Walter Reed, Washington, D.C.

LAB/BRANCH
Behavioral Biology Branch

SECTION
Section on Functional Neurochemistry

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

SUMMARY OF WORK (200 words or less - underline keywords)

Molluscan ganglia contain identified neurons which are excellent model systems for the analysis of neuronal regulatory mechanisms. The principal aim of this project is to study the biosynthesis, packaging, axonal transport, and release of polypeptides from these neurosecretory cells.

PHS-6040
(12-75)

Project Description:

Objectives: To study the biosynthesis, packaging, axonal transport, and release of specific polypeptides from identified, molluscan, neurosecretory cells.

Methods Employed: Conventional biochemical methods for the analysis of proteins and peptides are employed. In many cases, the conventional techniques must be scaled down to the microscale. Most notable in this regard is our use of the microgradient gel for electrophoresis (in collaboration with R. Ruchel). This gel system can separate proteins from fractions of a single cell with high resolution, and in the range from 10^6 to 10^3 daltons on a single gel.

Major Findings: Over the past few years we have demonstrated that very active biosynthetic and axonal transport systems associated with neurosecretion of polypeptides exist in specific identified neurons in molluscan ganglia (see refs. 1-7). This work has led to a model described in Loh et. al. (1975), which we have attempted to test during this past working period. Three specific issues were addressed: 1) Whether the specific low molecular weight polypeptides synthesized by these neurons, which are stored in abundance by the cells were 'water soluble', 2) whether autoradiographic kinetic analysis at the electronmicroscopic level would correspond to the biochemical kinetic data, and 3) whether subcellular granule fractions would be enriched in the putative secretory polypeptides.

The polypeptides could not be extracted in water by conventional lysis techniques. Therefore, a new 'osmotic lysis' technique utilizing ethylene glycol was developed. This method selectively extracted polypeptides sequestered in granules, and with it we could demonstrate that the specific neuronal peptides were water-soluble. Utilizing microgradient gels (discussed above), these water soluble peptides could be separated from individual neurons with a high degree of specificity, thus allowing for a more detailed analysis of the peptide heterogeneity in these cells. Preliminary autoradiographic data (E. Neale) in correlation with biochemical studies indicate that the low molecular weight polypeptides are, indeed, in neurosecretory granules. Further work is necessary in this area to determine the synthetic and packaging cycles in these cells, and their morphological correlates. Analysis of subcellular fractions, in collaboration with M. Daniels, confirmed that the peptides were associated with particulate (i.e., granules) material.

Radioimmunoassay experiments in collaboration with Drs. Straus and Yalow (VA Hospital, N. Y.) have shown that various mammalian peptide hormones are found in the blood of Aplysia. A report on gastrin has been published (ref. 8).

Significance to Biomedical Research and the Program of the Institute: The main significance of this work is that we have an opportunity in this model system to examine biochemical events in single neurons (i.e., in the soma) which are not accessible in more complex nervous systems. The data and approaches are directly transferrable to vertebrate systems (see project report on vertebrate peptides). Such studies provide a firm foundation for further studies of the cell biology of neuroendocrine systems, which are intimately involved in the growth and development of organisms.

Proposed Course: Collaborative efforts with Dr. E. Neale are in progress to examine the packaging of the polypeptides into granules in the neurosecretory cells. These experiments will continue to attempt to correlate electron microscopic autoradiography with the biochemical data.

Publications:

1. Gainer, H. and Barker, J. L. Selective modulation and turnover of proteins in identified neurons of *Aplysia*. *Comp. Biochem. Physiol.* 51B: 221-227, 1975.

2. Gainer, H., Barker, J. L. and Wollberg, Z.: Preferential incorporation of extracellular amino acids into neuronal proteins. *J. Neurochem.* 25: 177-179, 1975.

3. Gainer, H.: Behavioral regulation of biochemical and physiological properties of identified neurons. In Galun, R., Hillman, P., Parnas, I. and Werman, R. (Eds.): *Sensory Physiology and Behavior*. New York, Plenum Press, 1975, pp. 237-274.

4. Loh, Y. P. and Gainer, H.: Low molecular weight proteins in identified molluscan neurons. I. Synthesis and storage. *Brain Res.* 92: 181-182, 1975.

5. Loh, Y. P. and Gainer, H.: Low molecular weight proteins in identified molluscan neurons. II. Processing, turnover and transport. *Brain Res.* 92: 193-305, 1975.

6. Loh, Y. P., Sarne, Y. and Gainer, H.: Heterogeneity of proteins synthesized, stored, and released by the Bag cells of *Aplysia californica*. *J. Comp. Physiol.* 100: 283-295, 1975.

7. Loh, Y. P., Barker, J. L. and Gainer, H.: Neurosecretory cell protein metabolism correlated with diapause in the land snail, *Otala lactea*. *J. Neurochem.* In press.

8. Straus, E., Yalow, R. S. and Gainer, H.: Molluscan gastrin: Concentration and molecular forms. *Science* 190: 687-689, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00053-08 BB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Information processing in the central auditory system of mammals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. E. Alexander	Medical Officer	BB	NICHD
	J. D. Newman	Physiologist	BB	NICHD
	D. Symmes	Head, Section on Brain & Behav.	BB	NICHD

COOPERATING UNITS (if any)

Department of Primate Behavior, Max Planck Inst., Munich, Federal Republic of Germany

LAB/BRANCH

Behavioral Biology Branch

SECTION

Section on Brain and Behavior

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.3	.5

SUMMARY OF WORK (200 words or less - underline keywords)

Single unit recordings are made from neurons in the auditory system of squirrel monkey. Species typical vocalizations previously tape recorded are used as stimuli in a series of investigations aimed at disclosing systematic parameters of neural coding of this model language. Current work is focussed on comparisons between "primary" and "secondary" auditory cortical areas and on thalamic nuclei involved in auditory processing.

PHS-6040
(12-75)

Project Description:

Objectives: This project is concerned with the processing of complex sounds by the primate cerebral cortex and related brain structures. Special attention is being paid to sounds used by primates in intra-specific communication, to determine which cues are used by the brain and the animal to distinguish between the various elements of the communication repertoire.

Methods Employed and Major Findings: The major effort expended during FY 76 has involved analysis of recently collected data on response characteristics of neurons located in "secondary" auditory areas of the brain and comparison of these results with previously obtained data on "primary" auditory areas. The body of data is substantial - about 200 cells in the current sample and over 500 from previous experiments - and the differences between the processing mechanisms as revealed by single unit discharge patterns are subtle. It is hoped that a systematic classification of cell types found in different parts of the central auditory processor can be completed by the end of the year.

New studies involving the techniques of single cell recording have been initiated in late FY 76 based in part on the results of the analysis cited above and in part on data collected by Dr. Newman during his year of work in Munich. One study has the objective of characterizing neuronal discharges in post-arcuate frontal cortex, and may provide a clearer picture of the link between purely auditory and emotional-limbic processing. A second new study initiated this year is concerned with recording from the thalamic nuclei which project most directly to the superior temporal cortex (medial geniculate and medial pulvinar). An account of response properties in these relay and feedback nuclei will be of great value in developing a more comprehensive scheme of the manner in which species typical vocal information is encoded and transferred from purely auditory to other brain areas. Both of these studies are in the data collection phase and should be completed in approximately 6 months.

During the past year an initiative has been undertaken to expand anatomical knowledge of the squirrel monkey auditory system in a parallel manner to our physiological studies. Most of the anatomical data on which interpretation of our findings rests is based on old world monkeys, especially the rhesus. It is apparent that species differences exist in other sensory systems and the need for direct evidence in Saimiri is thus emphasized. These studies employ new technology involving double label injections of horseradish peroxidase (HRP) and radioactive amino acid (RAA) into auditory cortex, a method which has been successfully employed in several mammalian species. The method has the advantage of great efficiency of animal use (desirable in view of diminished availability of squirrel monkeys). HRP labels the afferent cells to the region of interest via retrograde transport, and RAA labels the afferent cells via anterograde transport.

Significance to Biomedical Research and the Program of the Institute: The studies completed comparing properties of "primary" and "secondary" auditory areas with respect to processing of both artificial and naturally occurring acoustic stimuli are unique and represent substantial additions to knowledge of brain functioning in the auditory realm. Because the animal model (squirrel monkey) possesses a rudimentary language with a vocabulary of known components and a syntax, it is anticipated that this knowledge will contribute to future studies on normal and abnormal language development.

Proposed Course: To continue characterization and systematic comparison of cortical and subcortical auditory regions in the squirrel monkey brain; to expand anatomical description of the relevant areas; to continue efforts to identify cells and response characteristics modifiable by experience; to develop technology for computer based simulation of natural monkeys calls.

Publications:

1. Newman, J. D. and Lindsley, D. F.: Single unit analysis of auditory processing in squirrel monkey frontal cortex. Exper. Brain Res. In press.

2. Newman, J. D.: Perception of sounds used in species-specific communication: The auditory cortex and beyond. In Goldsmith and Moor-Jankowski (Eds): Advances in Medical Primatology. New York, Plenum Press. In press.

3. Newman, J. D.: Single unit analysis of prefrontal cortex in primates. Exper. Brain Res. In press.

4. Ploog, D., Hupfer, K., Jurgens, U. and Newman, J. D.: Neuroethological studies of vocalization in squirrel monkeys with special reference to genetic differences of calling in two subspecies. In Brazier, M. A. B. (Ed.): Growth and Development of the Brain. New York, Raven Press, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00054-02 BB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structural and behavioral analysis of vocal communication in squirrel monkeys

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Lieblich	Staff Fellow	BB	NICHD
	J. D. Newman	Physiologist	BB	NICHD
	D. Symmes	Head, Section on Brain & Behav.	BB	NICHD

COOPERATING UNITS (if any)
Dr. Beverly White, NIAMD
Dept. of Primate Behavior, Max Planck Inst., Munich Federal Republic of Germany

LAB/BRANCH
Behavioral Biology Branch

SECTION
Section on Brain and Behavior

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland

TOTAL MANYEARS: 2.3	PROFESSIONAL: 1.8	OTHER: .5

SUMMARY OF WORK (200 words or less - underline keywords)

This project is concerned with 1) obtaining a detailed description of vocal behavior of squirrel monkeys, including the frequency of occurrence of defined microstructural features in a variety of behavioral contexts; and 2) delineating the relative contribution of genetic and environmental factors to development of normal vocal behavior. Both feral and laboratory reared monkeys are studied, and methods employed include spectrographic analysis, manipulation of social context, and formal behavioral training.

Project Description:

Objectives: This project is concerned with 1) obtaining a detailed description of vocal behavior of squirrel monkeys, including the frequency of occurrence of defined microstructural features in a variety of behavioral contexts; 2) delineating the relative contribution of genetic and environmental factors to development of normal vocal behavior in infant monkeys.

Methods Employed and Major Findings: Major attention this year was placed upon two aspects of vocal communication: ontogeny of vocal traits and communicative significance of vocal variability. Our ontogenetic studies have concentrated on infants born in our laboratory to females of a racial type ("Roman") which has not previously been studied utilizing our microstructural techniques. We have found in these infants as in those studied previously that vocal traits characteristic of their mothers are strongly present, and that in some instances still being studied paternal influences appear. We have also begun tracing the ontogeny of vocal traits of a hybrid infant born to parents from two genetically and phenotypically distinct *Saimiri* populations. The methods employed in this research include both conventional sound spectrograms and direct digital Fourier transform analysis via newly developed computer programs.

It should be noted that our in-house breeding program intended to generate infants having parents of known vocal characteristics and specifiable karyotypes produced a crop of healthy infants well below expectations. Considerable efforts have been devoted to isolating the factors contributing to these results with a high incidence of glomerular nephritis in our colony being the only clear pathology identified. Several steps have been taken or will be taken prior to the next breeding season aimed at improving the yield of young monkeys which can be characterized vocally. It should also be noted that difficulties in the early years of establishing breeding groups of new world monkeys have been reported from other laboratories.

Our studies this year on communicative significance of vocal variability have involved analysis of Twitter vocalizations produced during social interactions of adult females. Since our previous work had demonstrated that Twitters could be dissected into simpler constituent parts ("syllables"), we wished to investigate the extent to which rules of usage were followed by different individuals, and to learn to what extent individuals showed preferences in their usage of particular syllables and syllable combinations. About 700 Twitters were analyzed with the Voiceprint Sound Spectrograph. A revision of our earlier syllable classification has been devised, employing both frequency vs. time and amplitude characteristics of Twitters. Twelve different syllable types are sufficient to describe the twitters produced by all the monkeys studied to date. This study has resulted in the following findings: 1) there are certain rules of combining syllables that are followed by all individuals ("grammar"); 2) individuals have the capacity to modify their usage of specific syllables and syllable combinations ("plasticity"); 3) individuals additionally show syllable usage preferences which appear to be long term vocal traits. Since the Twitter call is known to develop to its adult form more slowly than other call types in the squirrel monkey repertoire and to exhibit plasticity in the adult we plan to study Twitter development in infants in the hope that an improved model of language acquisition in the infrahuman primate may be obtained.

Significance to Biomedical Research and the Program of the Institute: Squirrel monkeys possess one of the most specialized and discrete systems of vocal communication below man. Understanding both syntactical and semantic properties of this system would have wide implications for the fields of animal behavior and human psycholinguistics.

Proposed Course: Improvements in health screening and seclusion of breeding groups are incorporated in plans for in-house breeding of squirrel monkeys. A yield of 5-8 infants per year is desired, and will permit studies of mother-infant vocal recognition and behavioral modification of vocal output in young monkeys. It is planned to continue vigorously the microstructural analysis of selected vocalizations utilizing more efficient computerized techniques.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00055-01 BB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cryogenic depression of developing prefrontal cortex in primates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. E. Alexander	Medical Officer	BB NICHD

COOPERATING UNITS (if any)

P. S. Goldman, Psychology Laboratory, National Institute of Mental Health

LAB/BRANCH
Behavioral Biology Branch

SECTION
Section on Brain and Behavior

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

SUMMARY OF WORK (200 words or less - underline keywords)

The project involves the analysis of developing function in the immature primate prefrontal cortex. Infant and juvenile Rhesus monkeys are trained to perform a delayed response task which requires short term memory for positional cues. This task is known to be an indicator of prefrontal function in the mature animal. Utilizing local, reversible cooling adapted to chronic unanesthetized animals, we are able to examine the effects of temporary cyrogenic depression of prefrontal cortex at various times during each animal's period of maturation. Due to known connections between prefrontal cortex and two subcortical regions, the nucleus medialis dorsalis and the caudate nucleus, we are also examining the development of prefrontal influences on the latter two structures by the use of subcortical single unit recording in conjunction with prefrontal cooling. In addition to tracing of afferent and efferent connections of recording sites with immunoperoxidase localization of injected horseradish peroxidase and autoradiographic visualization of injected (^3H)-leucine, further work will include single unit recording from infant prefrontal cortex during behavioral performance.

Project Description:

Objectives: In this new project our intention is to examine the physiological mechanisms underlying the functional development of the prefrontal association cortex in Rhesus monkeys. We are further interested in delineating and analyzing the development of prefronto-subcortical interactions via well known pathways between prefrontal cortex and both the caudate nucleus and the nucleus medialis dorsalis. It is anticipated that the findings may contribute to our understanding of the development of higher cortical functions in general.

Methods Employed: Infant and juvenile Rhesus monkeys are trained to perform a delayed response (DR) task involving the transient memorization of a positional cue. The animals are implanted with devices utilized for localized reversible cooling of cerebral cortex, a technique which has been adapted for use with chronic, unanesthetized animals by several authors, including Dr. Alexander. Single unit recordings are being made from selected subcortical sites during cryogenic cortical depression, as well as during behavioral testing. In addition, neural pathways relevant to this study are being analyzed via orthograde transport of (^{3}H)-leucine visualized autoradiographically, and retrograde transport of horseradish peroxidase localized with immunoperoxidase techniques.

Major Findings: Using each animal repeatedly as its own control (over periods of testing lasting several months) we have confirmed the lack of dependence upon prefrontal cortex of DR performance during infancy, and have established an approximate age of 30 months as the point at which performance of this task becomes critically dependent upon intact prefrontal cortex. The pattern of neuronal firing in the caudate nucleus during DR testing has been determined in immature monkeys, which was of interest to us in view of the well-documented dependence of DR performance levels on the integrity of this structure from earliest infancy through adulthood. By means of cryogenic depression of prefrontal cortex during single unit recording from the caudate nucleus, we have found evidence for prefrontal influences on caudate neurons prior to the time that the animal becomes dependent upon prefrontal cortex for DR performance.

Significance to Biomedical Research and the Program of the Institute: We feel the strategies and techniques employed in this project offer several advantages in approaching the problem of development of higher cortical functions. The reversibility of the cooling technique allows each animal to act as its own control, eliminating the need for large numbers of subjects, and enhancing the reliability of the results. The brevity of the period during which cortical function is cryogenically depressed also minimizes the possibility of functional re-organization of remaining cerebral tissue. This affords the opportunity for a more straightforward interpretation of the effects of cortical cooling than is possible following brain lesions, where functional reorganization of the brain cannot be so readily discounted as an explanation for the observed effects. Because of the rapid induction and reversibility of the cooling effects, it has proven quite feasible to examine the influences of prefrontal cooling on neuronal activity in subcortical structures known to receive input from prefrontal cortex. This approach has already begun to elucidate the mechanisms by which the prefrontal cortex comes to assume an important role in behavior during its maturation. We are particularly interested in determining the physiological basis for the behavioral sparing which occurs with early prefrontal lesions, considering the obvious clinical relevance to brain damage in children.

Proposed Course: We have begun to explore development of prefrontal influences on neural activity in nucleus medialis dorsalis which, like the caudate nucleus, has known reciprocal connections with prefrontal cortex, and is necessary for DR performance from early infancy onward. We also intend to utilize variants of the DR task, such as delayed alternation, to refine the analysis of the functional development of the prefrontal cortex. Additional plans include the study of neuronal activity in developing prefrontal cortex.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00056-01 BB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of MSH synthesis and release

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y. P. Loh	Visiting Fellow	BB	NICHD
OTHER:	H. Gainer	Head, Section on Funct. Neurochem.	BB	NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Behavioral Biology Branch

SECTION
Section on Functional Neurochemistry

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	0

SUMMARY OF WORK (200 words or less - underline keywords)

The neurointermediate lobe of the toad, Xenopus laevis, synthesizes and releases melanocyte stimulating hormone (MSH). Lobes from dark adapted toads synthesize tenfold more protein and peptide (MSH) than light adapted toads. The release of MSH appears to be under the regulation of dopamine from the median eminence. Synthesis of β-MSH (and possibly the opiate peptide, endorphin) from a protein precursor in the intermediate lobe is actively under study.

PHS-6040
(12-75)

Project Description:

Objectives: To study the regulation of biosynthesis and release of MSH in the neurointermediate lobe of the toad, *Xenopus laevis*.

Methods Employed: Similar to methods described in other reports from this section.

Major Findings: Light or dark adaptation of the toad produces dramatic long-term increases in the release and synthesis of MSH from the neurointermediate lobe. These changes appear to be under the control of dopamine from the median eminence. Biosynthetic studies have shown that the MSH peptide is synthesized very rapidly by ribosomal mechanisms. Considerable further processing of putative protein precursors in the lobe occurs after the MSH is evolved, suggesting that other peptide products are produced by the system. One likely candidate is endorphine, the opiate peptide.

Significance to Biomedical Research and the Program of the Institute: Peptides such as α- and β-MSH have been implicated in higher brain functions (e.g., avoidance learning). While MSH is clearly important for melanophore control in amphibia, no clear function has emerged for this peptide in mammals. Studies on this peptide may provide insight into its functional significance. In addition, the neurointermediate lobe is under profound control by nerve terminals (biogenic amines) from the hypothalamus, and represents an excellent model system for the study of neural regulation of release and synthesis of a peptide messenger.

Proposed Course: Attempts will be made to isolate the protein precursor of MSH by isolation of the m-RNA and its translation in an *in vitro* (wheat germ) system. Dark adaptation should produce lobes with abundant m-RNA. In addition, the regulatory actions of biogenic amines (e.g., dopamine, serotonin, etc.) will be investigated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00057-01 BB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Axonal proteins: biosynthesis, transport and function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Gainer	Head, Section on Funct. Neurochem.	BB	NICHD
OTHER:	Y. Sarne	Visiting Fellow	BB	NICHD
	E. Neale	Staff Fellow	BB	NICHD
	B. K. Schrier	Medical Officer	BB	NICHD

COOPERATING UNITS (if any)

I. Tasaki, H. Pant and I. Inoue, Laboratory of Neurophysiology, NIMH
R. J. Lasek, Case-Western Reserve Univeristy

LAB/BRANCH

Behavioral Biology Branch

SECTION

Section on Functional Neurochemistry

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0

SUMMARY OF WORK (200 words or less - underline keywords)

Giant axons in the crayfish and squid have been studied. In both systems evidence for the substantial transfer of newly synthesized macromolecules (proteins) from Schwann cells (glia) into axons has been obtained. In the case of crayfish motoraxons, the glia appear to be a sufficient source of axonal proteins to maintain the axon (including transmitter enzymes). The proteins on the inner surface of the squid giant axon membrane have been studied using membrane labeling techniques. A correlation between the presence of specific proteins on the membrane and excitability of the axon has been found.

Project Description:

Objectives: To study the biosynthetic origins of axoplasmic proteins, and their functions in axonal excitability and transport.

Methods Employed: Protein biochemical techniques, autoradiography, and membrane protein labeling techniques.

Major Findings: Axoplasm does not contain ribosomal RNA, and is not capable of *de novo* protein synthesis. Our studies have shown that, in addition to axonal transport from the cell body, the glia surrounding the axon are very active in protein synthesis and transfer a wide variety of macromolecules (i.e., newly synthesized proteins) to the axon (see Lasek, *et. al.*, *PNAS*, 71, 1188-1194, 1974). Recently, we have shown that in crayfish motor axons, which do not degenerate and remain functionally intact following axotomy, the maintenance of the axon is virtually under complete control by the glial investment. In addition, studies on the structural proteins of the squid giant axon suggest that tubulin may be a glycoprotein in axons. This might implicate glycosylation in axonal transport. In collaboration, with I. Tasaki's laboratory at the Marine Biological Laboratory, we have found the excitability of the axon is related to the presence of specific proteins on the inner surface of the axon membrane. Depolarization of the axon causes a calcium dependent release of these proteins.

Significance of Biomedical Research and the Program of the Institute: This definitive demonstration that macromolecules can be transported from glia to neurons, represents the first example of such metabolic cooperation between cells in the nervous system, *in situ*. Such a mechanism may underly trophic interactions, and be a basis for inter-cellular communication during development.

Proposed Course: Further efforts to purify axonal tubulin from the squid axon will be necessary to unequivocally determine whether this protein contains carbohydrate. If this is indeed the case, then an analysis of the carbohydrate residues will be done. In analogy with immunological systems, the carbohydrate components on tubulin may represent recognition sites for axonal transport. Probably the most exciting issue, is the membrane protein(s) associated with excitability. Further work utilizing other membrane protein labels and intracellular perfusion will be necessary to identify these proteins which may be fundamental regulatory agents for excitability.

Publications:

1. Gainer, H. and Gainer, V. S.: Proteins in the squid giant axon. In Reuben J. R. and Purpura, D. (Eds.): *Electrobiology of Nerve Muscle and Synapse*. New York, Raven Press. In press.
2. Barker, J. L., Hoffman, P. N., Gainer, H. and Lasek, R. J.: Rapid transport of proteins in the sonic motor system of the toadfish. *Brain Res.* 97: 291-301 (1975).

3. Pant, H. C., Tasaki, I. and Gainer, H.: Glycoproteins in the squid axon. Comp. Biochem. Physiol. In press.

4. Sarne, Y., Neale, E. A. and Gainer, H.: Protein metabolism in transected peripheral nerves of the crayfish. Brain Res. In press.

5. Sarne, Y., Schrier, B. K. and Gainer, H.: Evidence for the local synthesis of a transmitter enzyme (glutamic acid decarboxylase) in a crayfish peripheral nerve. Brain Res. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00058-01 BB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biosynthesis of peptides in the vertebrate nervous system

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Gainer	Head, Section on Funct. Neurochem.	BB	NICH
	Y. Sarne	Visiting Fellow	BB	NICH

COOPERATING UNITS (if any)
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LAB/BRANCH
Behavioral Biology Branch

SECTION
Section on Functional Neurochemistry

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

SUMMARY OF WORK (200 words or less - underline keywords)

Experiments on rats show that following injection of S^{35}-cysteine into the supraoptic nucleus, labeled oxytocin, vasopressin, and neurophysin appear first in the nucleus, then in the median eminence (1 hour), and finally in the posterior pituitary (> 1.5 hour). The peptides and neurophysin are synthesized first as a protein precursor(s) (mw, 20,000 daltons) and the enzymatic degradation of the precursor appears to occur intra-granularly during axonal transport.

Project Description:

Objectives: To determine whether the neurohypophysial peptide hormones, oxytocin and vasopressin, are synthesized as prohormones (protein precursor), and to study the cellular mechanisms underlying this process. This vertebrate neurosecretory system is ideal as a model for the development of necessary techniques and approaches for the study of peptidergic neurons and systems in general. The latter represents a major long-term objective of this section.

Methods Employed: Gel electrophoresis, thin-layer chromatography, high-voltage electrophoresis, radioisotope technology, stereotaxic intracerebral micro-injection, and isolation of brain nuclei for analysis.

Major Findings: The major finding in this work is the direct demonstration that a 20,000 dalton protein synthesized in the supra-optic nucleus of the rat is the precursor for the neurohypophysial hormones, and that this precursor molecule(s) appears to be packaged in neurosecretory granules, and is processed to the peptides and neurophysin during axonal transport. When the rats are given 2% NaCl in place of water to drink (7 days) there is a 5-10 fold increase in precursor synthesis, and labeled oxytocin, vasopressin, and neurophysin in the posterior pituitary.

Significance to Biomedical Research and the Program of the Institute: The finding that oxytocin and vasopressin are synthesized first as prohormones places these neuronal peptides in the same category as other peptide hormones (e.g., pro-insulin, insulin). Perhaps more significant is that the experimental paradigm first developed for molluscan identified neurons is highly effective as an approach for peptide biosynthesis in vertebrate systems. Such peptidergic neurons and systems in the vertebrate brain may have important regulatory (e.g., trophic) and integrative roles during development.

Proposed Course: To isolate and chemically characterize the protein precursor(s) of oxytocin and vasopressin (e.g., amino acid analysis and sequence). To determine if specific enzymes are involved in the processing. To examine the mechanisms which cause an increase in synthesis during osmotic stress of the rats. In addition, other peptides (e.g., substance P) and nuclei (e.g., substantial migra, habenular, etc.) will be studied using the strategy evolved from this study.

Publications:

1. Gainer, H.: Peptides and neuronal function. In Costa, E. (Ed.): Advances in Biochemical Pharmacology. New York: Raven Press. In press.

Project Description:

Objectives: To determine the cellular mechanisms of clinically important pharmacologic agents using various neuronal preparations as model systems.

Methods Employed: Three types of preparation have been used: 1) isolated invertebrate central nervous system; 2) isolated frog spinal cord; 3) mouse central nervous system grown in tissue culture. The preparation is bathed in the appropriate saline and one or two microelectrodes placed in single, identified nerve cells. Neurotransmitters and drugs are either bath applied or microiontophoresed and resting membrane properties or synaptic events assayed. Such preparations permit simple or sophisticated examination of drug effects under well-controlled conditions.

Major Findings: Non-narcotic analgesics (salicylates, mefenamates, acetanilides) all increase resting K^+ conductance relative to resting Cl^- conductance in invertebrate neuronal membranes in a dose-dependent, reversible manner. These changes in membrane properties depress neuronal excitability. The drug effects can be qualitatively and quantitatively correlated with non-narcotic analgesia in the vertebrate. The biophysical mechanisms underlying the drug effects appear to be related to an increase in the anionic field strength of the membrane. Drug-induced changes in membrane properties are highly correlated with the hydrophobicity of the drug, leading to the conclusion that the negatively charged, hydrophobicity drug non-specifically partitions into neuronal membranes thereby increasing their anionic field strength of the membrane.

Similar results have been obtained with a series of agents which uncouple oxidative phosphorylation in mitochondria, leading to the hypothesis that these agents uncouple metabolism by partitioning into mitochondrial membranes and increasing H^+ permeability.

Convulsants (strychnine, picrotoxin and bicuculline) have been applied to sensory afferent terminals and motoneurons in the isolated frog spinal cord. All three initiate convulsive activity, the magnitude of which is dose-dependent. All three convulsants have specific antagonistic actions on the effects of putative inhibitory neurotransmitters. Picrotoxin (and bicucculline) blocks the actions of β-alanine, taurine and γ-aminobutyric acid (GABA), but not glycine. Strychnine blocks the actions of glycine, β-alanine and tuarine, but not GABA. Thus, at least three distinct populations of receptors for inhibiting transmitter are present in the amphibian spinal cord. The convulsants appear to be acting primarily by selectively blocking inhibitory transmissions, thus enhancing in a relative manner, excitatory synaptic events.

The effects of general anesthetics (pentobarbital, chloralose, chloroform, urethane, and ethanol) and one anticonvulsant (diphenylhydantoin) on neuronal membrane and synaptic events have been examined using molluscan nervous systems *in vitro* and mouse nervous system in tissue culture. Most of the research has been carried out with pentobarbital which selectively depresses excitatory transmissions in invertebrate preparations, as well as responses to excitatory

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00059-01 BB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Pharmacologic studies of vertebrate and invertebrate neurons

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Barker	Medical Officer	BB NICHD
OTHER:	B. R. Ransom	Medical Officer	BB NICHD

COOPERATING UNITS (if any)
H. Levitan, Department of Zoology, University of Maryland, College Park, Md.

LAB/BRANCH
Behavioral Biology Branch

SECTION
Section on Neurobiology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

SUMMARY OF WORK (200 words or less - underline keywords)

This project has concentrated on examining the neuronal bases for drug action by using electrophysiological recordings from a variety of in vitro preparations which provide useful models of the mammalian nervous system for studying the membrane mechanisms underlying the actions of clinically important drugs. The drugs investigated include 1) non-narcotic analgesics, 2) mitochondrial uncoupling agents, 3) convulsants and 4) general anesthetics. The primary observations are 1) non-narcotic analgesics depress neuronal excitability by non-specifically partitioning into the cell membrane; 2) mitochondrial uncoupling agents reversibly enhance cation permeability of membranes by non-specifically partitioning into membranes; 3) convulsants increase excitability by antagonizing specific effects of inhibitory neurotransmitters; 4) general anesthetics depress excitability by decreasing excitatory neurotransmitter effects and increasing inhibitory neurotransmitter actions. The results indicate that different classes of neuropharmacologically important agents act upon different neuronal membrane mechanisms and that model invertebrate and vertebrate systems are useful in elucidating mechanisms of drug action at the single cell level.

putative neurotransmitters. Inhibitory transmissions are preserved. The other agents tested also selectively depress excitatory transmitter effects, specifically those associated with the commonest receptor-coupled conductance change (Na^+-K^+). Excitations resulting from activation of other conductance changes are not depressed nor are various forms of inhibition. The depression of excitation is highly correlated with the hydrophobicity of the agent and with its general anesthetic potency in the vertebrate.

The inhibitory effects of pentobarbital on mouse neurons in tissue culture are three-fold: 1) depression of the excitatory effects of the putative excitatory neurotransmitter glutamate; 2) specific enhancement of the inhibitory conductance change produced by GABA and induction of another phase of the GABA response without similar changes in the inhibitory response to another inhibitory transmitter glycine and 3) direct inhibitory effects of pentobarbital on neuronal membrane properties. The results extend the observations in the invertebrate and complement work in other laboratories showing a selective depression of excitatory synaptic transmission by anesthetics.

Significance to Biomedical Research and the Program of the Institute: Study of the cellular basis of neuropharmacologically active agents is *a priori* important in determining how clinically useful drugs act on the nervous system. Most, if not all of the neuroactive agents which are presently employed in clinical practice are used empirically. Understanding mechanisms of action will aid in their usage and in further development of safer drugs. A dividend of this research is increased understanding of mechanisms underlying neuronal excitability as well as the use of particular agents for investigating relatively specific membrane mechanisms.

Proposed Course: Further analysis of data already acquired and more sophisticated approaches to studying drug effects at the membrane level are planned.

Publications:

1. Barker, J. L.: CNS depressants: Effects on post-synaptic pharmacology. *Brain Research* 92: 35-55, 1975.

2. Barker, J. L: Divalent cations: Effects on post-synaptic pharmacology of invertebrate synapses. Brain Research 92: 307-323, 1975.

3. Barker, J. L.: Inhibitory and excitatory effects of CNS depressants at invertebrate synapses. *Brain Research* 93: 77-90, 1975.

4. Barker, J. L.: The neuropharmacology of general anesthesia. Scientific American. In press.

5. Nicoll, R. A., Padjen, A. and Barker, J. L: Analysis of amino acid responses on frog motoneurons. *Neuropharmacology*. In press.

6. Barker, J. L., Nicoll, R. A. and Padjen, A.: Factors underlying the genesis of dorsal root potentials in the frog spinal cord. *J. Physiol.* In press.

7. Barker, J. L. and Levitan, H.: Mitochondrial uncoupling agents: effects on membrane permeability of molluscan neurons. J. Mem. Biol. 25: 361-380, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00060-01 BB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Physiological roles of peptides in the nervous system.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Barker	Medical Officer	BB	NICHD
OTHER:	None			

COOPERATING UNITS (if any)
T. G. Smith, Laboratory of Neurophysiology, NINCDS
K. Futamachi, Laboratory of Neurophysiology, NINCDS
Y. Grimm-Jorgensen, Dept. of Anatomy, Univ. of Connecticut Medical School

LAB/BRANCH
Behavioral Biology Branch

SECTION
Section on Functional Neurochemistry

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to investigate the physiological roles of peptides in the nervous system, employing both invertebrate and vertebrate systems. Much of the work has been done on molluscan nervous systems, examining the electrophysiological effects of the vertebrate peptide vasopressin and analogues on membrane properties of neurosecretory cells. The peptide produces long-lasting changes in the membrane properties of specific cells characterized by the induction or enhancement of bursting pacemaker potential activity. The peptide effects are unlike those caused by conventional neurotransmitters and appear to reflect a neurohormonal role rather than a neurotransmitter role. Results obtained on the invertebrate may provide insight into one of the ways peptides may act in the mammalian central nervous system as long-term regulators of neuronal excitability over a defined population of nerve cells. A second aspect of the work has focussed on the distribution of tripeptide thyrotropin releasing hormone (TRH) in invertebrate and vertebrate nervous systems. That this peptide is present in species devoid of pituitary tissue and is located throughout the nervous system including the spinal cord strongly suggests functional roles for the peptide other than the release of thyroid stimulating hormone from the pituitary.

PHS-6040
(12-75)

Project Description:

Objectives: The project is concerned primarily with investigation of the physiological roles of peptides in neuronal function.

Methods Employed: The nervous systems of several molluscan species are isolated, bathed in saline and standard electrophysiological techniques employed for measuring membrane properties. In addition, voltage clamp methods are utilized to provide a more sophisticated analysis of membrane excitability. Peptides have been applied either in the bathing medium or by iontophoresis from micropipettes filled with concentrated solution. For TRH determination, the nervous system of invertebrates or vertebrates is carefully dissected, immediately frozen and then assayed for TRH activity by radioimmunoassay. TRH content is expressed per milligram protein.

Major Findings: Application of the vasopressin peptide rapidly leads to an increase in neuronal excitability with subsequent initiation of bursting pacemaker potential activity in specific molluscan neurons. The increased activity, manifest by an increased number of action potentials per unit time, long outlasts the period of peptide application. Threshold for the effect is nanomolar, which is several orders of magnitude less than threshold for the transient effects of neurotransmitter which can be obtained on the same cell. Associated with the induction of bursting pacemaker activity is a progressive prolongation of spike duration during the burst so that the last spike in a burst is twice as long as the first spike. Thus, the increase in excitability is considerably more than a simple increase in spike frequency. Voltage clamp analysis reveals that the membrane properties of peptide-sensitive cells in the non-bursting mode consist of a steady-state current voltage (I-V) relationship which is always positive. The effect of the peptide is to change the profile of the steady-state I-V curve so that it is now N-shaped, with a region of negative slope. The negative slope appears to be due to a voltage-dependent Na^+ conductance which inactivates slowly, if at all. This conductance provides the driving force to sustain the depolarizing phase of the bursting pacemaker potential. The hyperpolarizing phase of the pacemaker potential is due to activation of a voltage-dependent K^+ conductance which inactivates slowly. The peptide appears to increase the time constant of decay of this latter conductance. Thus, the peptide substantially alters the basic voltage-dependent membrane properties of the cell, unlike neurotransmitters whose actions are directed at voltage-independent membrane events. The molecular mechanisms underlying the peptide effects have not been investigated. The peptide may remain bound to the cell for a long period or may cause long-lasting changes in membrane peroperties. Of interest is the finding that removing extracellular Ca^{++} has qualitatively similar effects on all of the membrane properties assayed, suggesting that the peptide may work by regulating Ca^{++}'s modulation of the membrane properties important for generating pacemaker potentials. Three possible functional effects of the peptide are envisioned: 1) pulsatile, facilitated release in product, transmitter or hormone; 2) change in input-output relations of the cells from linear to non-linear; 3) increase in Ca^{++} influx into the cell body with possible consequences for synthesis and transport of low molecular weight proteins.

TRH has been found unevenly distributed in the nervous systems of several molluscan species, which are devoid of pituitary tissue. The amount present is similar to that found outside the hypothalamus of vertebrates and varies as a function of the state of activity of the mollusc. TRH is also unevenly distributed in the nervous system of the frog, considerable quantities being present in various parts of the central nervous system. The peptide is also present in the spinal cord, the concentration declining in a rostrocaudal direction. It is not present outside the central nervous system. These results suggest a possible functional role for TRH as a neurochemical.

Significance to Biomedical Research and the Program of the Institute: The possibility that peptides play physiological roles in neuronal function has long been suggested but only recently begun to be supported by various types of evidence. The research reported here is the first piece of strong evidence that one of the functional roles peptides may play is neurohormonal. Some peptides may thus belong to a class of neurochemical mediating cell-cell interactions that is operationally distinct from neurotransmitters. Peptide neurohormones appear to be operating on a broader plane of integration of neuronal function, regulating excitability over an extended period and through mechanisms different than those utilized by transmitters. The importance of understanding the physiological functions of peptides in the nervous system is clear. Disturbances in peptide functioning may underlie various behavioral, endocrine, and motor pathologies associated with central nervous system functioning. This research is a first step in our understanding of how peptides may operate at the cellular level.

Proposed Course: Analysis of data already obtained as well as continued experiments on peptide effects on various neuronal systems will build upon the foundation already laid.

Publications:

1. Barker, J. L.: Peptide regulation of neuronal excitability. Physiol. Reviews. In press.

2. Barker, J. L. and Smith, T. G.: Peptide regulation of neuronal membrane properties. Brain Res. 103: 167-170, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00061-01 BB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Early events in synapse formation in tissue culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S. A. Cohen Medical Officer BB NICHD

COOPERATING UNITS (if any)
Dr. Gerald D. Fischbach, Dept. of Pharmacology, Harvard Medical School
Dr. Francois Reiger, Dept. of Neurosciences, Children's Hosp. Med. Center, Boston
Dr. Susan Hauser, Section on Technical Development, NINCDS/NIMH

LAB/BRANCH
Behavioral Biology Branch

SECTION
Section on Neurobiology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

SUMMARY OF WORK (200 words or less - underline keywords)

Tissue culture systems have been developed in which neuromuscular junctions form rapidly de novo. Using high resolution Nomarski optics and electrophysiologic techniques the development and distribution of acetylcholine receptors on the muscle surface membrane have been continuously monitored on single cells during early stages of synapse formation. A small computer has been used to detect the first signs of neuromuscular transmission. Also the role that innervation plays on the expression of different molecular forms of acetylcholinesterase has been studied.

Project Description:

Objectives: To investigate the molecular mechanisms operative in the earliest stages of synapse development. Initial emphasis has focussed on analysing the nature of the interaction between nerve and muscle cells which regulates the distribution of acetylcholine (ACh) receptors in the muscle cell surface membrane and the expression of different molecular forms of acetylcholinesterase (AChE).

Methods Employed: Initially dissociated mononucleated myoblasts from chick embryos have been grown on collagen coated glass coverslips alone or co-cultured with small fragments (explants - approximately 0.5 mm square) of spinal cord. The cells were viewed at high magnification (500-1200x) on the stage of an inverted microscope equipped with Nomarski differential interference contrast optics. This optical system has a narrow depth of field and therefore allows visualization of fine nerve processes as they first contact and grow along the surface of muscle fibers. Intracellular microelectrode recordings of the muscle were made to measure the cells' responses to extracellular electric stimulation of contacting nerve processes or the micro-iontophoresis of ACh on the muscle cell membrane. Receptor distribution was also determined with autoradiographs of cultures exposed to ^{125}I-α-bungarotoxin - a ligand which binds specifically and irreversibly to nicotinic cholinergic receptors.

Major Findings: When spinal cord explants are added to already established muscle cultures functional neuromuscular junctions form on 70% of those muscles contacted by nerve processes within 24 hours and on some fibers as early as 7 hours. Transmitter is released from these simple synapses in quantal packets.

Sites of transmitter release have been identified and localized to within 5-8 μ in several ways. In each case a relative peak ("hot spot") of ACh sensitivity 3-10 x background values was found on the immediate subsynaptic muscle membrane. This is at an age when noninnervated muscle fibers have relatively few hot spots. Both the number of hot spots and the background ACh sensitivity was increased compared to controls soon after synapse formation. Functional innervation, not just nerve-muscle contact, was needed to induce the increase. To determine whether the nerve induces the hot spots at the transmitter release sites we have used a small computer to analyze sub-noise level signals. In this manner normally undetectable synaptic responses of 40-100 μV in amplitude have been recorded at the very earliest time the nerve processes contact the muscle. Evidence suggests that these small signals result from a normal sized quantum of transmitter released over muscle membranes with a low density of ACh receptors.

Studies have begun to analyze the cellular mechanisms responsible for the increased background ACh sensitivity of recently innervated muscle fibers. For example, preliminary results suggest that neither functional receptors nor transmission are required.

In other early experiments the development of AChE with synapse formation has been investigated. The overall amount of AChE is 2-3 x greater and a specific molecular form is 10-fold larger in combined spinal cord explant-muscle cultures than the sum of that in separate explant and muscle plates.

Other investigators in this laboratory (see Project No. Z01 HD 00046-07 BB) have found that cholinergic synapses form between a cell line of neuroblastoma X glioma hybrids and mouse muscle fibers in culture. I have repeated some of their experiments using chick instead of mouse muscle which may offer some advantages. Namely, synapses appear to form more rapidly with the chick muscle thereby making continuous electrophysiologic motitoring more feasible. More importantly, pure cultures of "naked" chick muscle fibers devoid of background cells and connective tissue can be obtained (unlike for the mouse, as of yet) thus increasing the resolution of visualization and microphysiology and allowing for potentially more meaningful biochemical analysis of cell specific molecular mechanisms.

Significance to Biomedical Research and the Program of the Institute: Knowing the mechanisms involved in cell-cell interactions and specificity of connectivity is crucial to understanding how the nervous system develops. Only then can we hope to recognize alterations of those mechanisms which may be responsible for disease. In the tissue culture systems described, for example, one has the potential to identify the specific molecular events signalling both the increased ACh receptor accumulation at synaptic sites and the suppression of receptors extrasynaptically. These systems should ultimately prove to be a useful model for studying the deficient functional control mechanisms manifest in pathologic states.

Proposed Course: Further elaboration of the steps involved in the redistribution of muscle ACh receptors with synapse formation is planned. Determining how the association between a hot spot of ACh sensitivity and transmitter release site obtains will be important. Questions of the stability of the development into mature neuromuscular junctions will be addressed. Effects of prolonged time in culture and activity on this development as well as on both junctional and extrajunctional ACh receptors will be examined.

Publications:

1. Hauser, S. and Cohen, S.: An isolated current monitor for iontophoresis. In Rhodine, C. N. (Ed.): Proceedings of the Thirteenth Annual Rocky Mountain Bioengineering and Thirteenth International ISA Biomedical Sciences Instrumentation Symposium. Pittsburgh, Pa.: Instrument Society of America. In press.

NICHD Annual Report
July 1, 1975 through June 30, 1976
Laboratory of Biomedical Sciences
National Institute of Child Health and Human Development

The Laboratory of Biomedical Sciences conducts research in the biochemistry of development, both normal and pathological.

In the *Section on Developmental Enzymology* interest centers on the purification and characterization of placental enzymes, pregnancy-associated enzymes in maternal plasma, and fetal enzymes. This basic enzymology will stimulate progress in the following areas of clinical research: (1) the evaluation of placental function by measuring pregnancy-associated enzyme activities in maternal plasma, (2) the elucidation of the biochemical basis for the clinical manifestations of errors of intermediary metabolism (currently, of glycogen and of lysine), and (3) the measurement in plasma of the activities of placental enzymes, produced ectopically by nontrophoblastic tumors, for the diagnosis of neoplasia and for monitoring the response to therapy.

Placental enzymes currently under consideration are heat-stable alkaline phosphatase and enzymes involved in the metabolism of glycogen and of amino acids. The progress in each of these areas of investigation is summarized in the following three paragraphs.

In a study of the control mechanism of gene expression in choriocarcinoma cells, alkaline phosphatase was found to be inducible by a variety of physiological and nonphysiological agents such as dibutyryl cyclic AMP, 5-bromodeoxyuridine, and inhibitors of DNA synthesis. The induction requires both RNA synthesis and protein synthesis. The induced enzyme does not differ substantially in heat stability and kinetic properties from heat-stable alkaline phosphatase of term placenta. The frequent occurrence of derepression of placental alkaline phosphatase in nontrophoblastic tumors *in vivo*, considered together with the ease with which derepression can be achieved *in vitro*, suggests that the ectopic presence of the enzyme may serve as an early signal of malignant transformation.

With respect to glycogen metabolism: glycogen synthetase from choriocarcinoma cells was found to resemble in many respects the enzyme from normal placenta, but it does have a K_a for glucose-6-P which is 5 to 10 times greater than the K_a of the placental enzyme; a unique regulatory mechanism for D-form glycogen synthetase, through activation by $MnSO_4$ and by Mn^{++}, has been discovered; placental alkaline phosphatase has been proved to be a phosphoprotein phosphatase, one substrate of which is glycogen synthetase; multiple forms of cyclic AMP-independent phosvitin kinase have been separated and characterized. These studies will contribute to a better understanding of fetal carbohydrate metabolism, which is greatly influenced by the placenta before the fetal liver has fully developed.

In the area of amino acid metabolism, two enzymes have been discovered which mediate the first steps in an alternate route for the metabolism of intermediates of lysine degradation. This new route provides the basis for a model that may give insight into the biochemistry of several recently discovered defects in the intermediary metabolism of lysine.

The Section on Physiological Controls has two research groups respectively examining endocrine and neuroendocrine mechanisms.

I. Studies are conducted on endocrine and nutrient control of cellular metabolism during development. Physiological experiments focus on glucose homeostasis in the perinatal period and development of fetal liver enzymes regulating glycogen metabolism and gluconeogenesis. Biochemical studies center on regulation of protein and nucleotide phosphotransferases and phosphatases and on defining the mode of action of glucocorticoids.

Studies on glucose homeostasis and regulation of glycogen-cycle enzymes in perinatal monkey and rat liver have documented extensively that galactose amplifies hepatic glucose release during hypoglycemia and glycogen synthesis during hyperglycemia.

Glucocorticoids markedly increase galactose-dependent glucose release in isolated fetal liver explants, and this correlates with increased glucose-6-phosphatase activity.

Two heat-stable protein inhibitors of phosphorylase phosphatase have been partially purified from rabbit skeletal muscle, the activity of one being regulated by phosphorylation by the cyclic AMP-dependent protein kinase.

Naturally occurring polyamines markedly stimulate pig-liver nucleoside diphosphate kinase.

A rapid two-stage procedure using DNA-cellulose chromatography has been developed which results in an 1800-fold purification of the hepatic glucocorticoid-receptor complex.

II. Studies are conducted on neuroendocrine mechanisms in two general areas of mammalian physiology: the pineal gland and the pituitary gland. Studies on the pituitary gland have concentrated on the effects of secretions of the pineal gland on pituitary function.

Investigations on the pineal gland have found, first, that the regulation of the enzyme which governs melatonin synthesis, N-acetyltransferase, is strongly influenced by temperature. This suggests that in addition to being a neuroendocrine phototransducer, the pineal gland may also be a neuroendocrine thermotransducer. Second, studies have provided evidence that during times of stress the pineal gland is not adrenergically stimulated substantially by circulating catecholamines because the pineal nerves take up catecholamines and thus preclude the accumulation of these compounds in the pineal perivascular space. Third, collaborative efforts have resulted in the development of a system for culturing pineal cells. Work in this laboratory has improved this preparation and has characterized the adrenergic-cyclic AMP regulation of pineal N-acetyltransferase activity. Fourth, collaborative studies using 2-fluoro-L-histidine have found that this amino acid not only inhibits the induction of pineal N-acetyltransferase activity but that it also inhibits the induction of several other enzyme systems. These include the steroid induction of liver tyrosine amino transferase and the benz[a]pyrene induction of liver-cell aryl hydrocarbon hydroxylase.

Investigations on the pituitary gland have, first, extended the characterization of a neonatal rat-pituitary organ-culture preparation. It is now clear that the LRF-stimulation of LH secretion by this preparation is specific in that another hypothalamic releasing factor, TRF, has no effect on LH secretion and that LRF has no effect on the release of TSH. Second, it was discovered that melatonin can inhibit LRF-stimulation of LH-release. The specificity of the inhibitory effects of melatonin have been investigated. Several similar compounds have been found to be either ineffective or effective only at 100-fold greater concentrations. In contrast, it was discovered that serotonin was active at low concentrations but that it could not inhibit LRF-stimulation of LH-secretion more than 50%. Third, studies *in vivo* using neonatal animals have indicated that melatonin can inhibit the LRF-stimulation of LH secretion, which confirms *in vitro* observations made by this group. Fourth, collaborative studies with 6-fluoromelatonin and 4,6-difluoromelatonin have indicated that these compounds are as potent as or more potent than melatonin in inhibiting the stimulatory effects of LRF on LH secretion. Fifth, analysis of pineal culture medium which had been fractionated by thin-layer chromatography has indicated that the anti-LRF activity of such medium is associated with serotonin and melatonin.

Investigators in the *Section on Intermediary Metabolism* develop laboratory analogs of mental retardation through the use of specific chemical agents and enzyme inhibitors, investigate the biosynthesis and degradation of macromolecules in neural tissues, study the mechanism of hydroxylation reactions of neural interest, and engage in studies on the mechanism of action of nerve growth factor. The studies of the Section range from the classical biochemical investigations of enzymes of neurochemical interest, and pharmacological efforts to inhibit or stimulate these enzymes, to behavioral experiments on animals in which the activity of such enzymes has been altered or inhibited. In this way, we hope to provide information on the basic biochemistry of the brain as well as on the conditions which alter the normal brain functions, such as various genetically transmitted mental retardation syndromes.

During the past year, most of the effort in the group has been devoted to the various aspects of the nerve growth factor problem. It has been demonstrated that changes in cyclic AMP levels are produced by the addition of nerve growth factor to superior cervical ganglia in organ culture and to isolated vesicles from adrenal medulla. Further, it has been shown that the addition of dibutyryl cyclic AMP to ganglia produces an increase in tyrosine hydroxylase levels similar to, but not as large as, that found upon addition of nerve growth factor. An immunological approach has allowed the direct measurement of tyrosine hydroxylase synthesis in these ganglia and to an assessment of the effect of nerve growth factor, cyclic AMP, and a number of other agents which act on the system. The complete purification of the antibody to the nerve growth factor by affinity chromatography has provided a new and selective tool for the study of the action of the nerve growth factor in isolated ganglia and in other systems. The change in tyrosine hydroxylase activity produced by nerve growth factor in ganglia in culture provides a sensitive assay for the measurement of nerve growth factor activity. On another level of organization it has been possible to grow isolated sympathetic neurons in the presence of primary cultures from dissociated pineal glands. The neurons survive in the absence of added nerve growth factor, and no nerve growth factor has been detected in the media from these cultures. The use of the purified antibody should provide information on the nature of the principle which supports the growth of the neurons.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00086-06 LBS

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Developmental Enzymology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Laboratory of Biomedical Sciences

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NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0.0

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is the purification and characterization of placental enzymes, pregnancy-associated enzymes in maternal plasma, and fetal enzymes. Topics of current interest are: (1) the induction in choriocarcinoma cells of placental alkaline phosphatase, an enzyme which appears in maternal serum during pregnancy and which is one of the ectopically produced proteins seen in certain patients with malignant neoplasms; (2) the phosphoprotein phosphatase activity exhibited by placental alkaline phosphatase; (3) the regulation of glycogen synthesis in human placenta and choriocarcinoma cells through phosphorylation and dephosphorylation reactions involving glycogen synthetase, phosphorylase, and various protein kinases and phosphoprotein phosphatases; (4) the elucidation of the enzyme reactions involved in lysine metabolism and of the relationship of these enzymes to the metabolic defects and clinical manifestations of hyperlysinemia, saccharopinuria, α-aminoadipic aciduria, and glutaric aciduria.

Project Description:

Objectives: The main objectives of this project are: (1) to study qualitative and quantitative changes in selected enzyme systems in the developing placenta and fetal organs in normal gestation; (2) to study qualitative and quantitative changes in these same enzyme systems in the developing placenta and fetal organs in reproductive disorders; (3) to isolate, purify, and characterize enzymes from the normal human placenta and to elucidate their relationship to heritable disorders of metabolism; and (4) to identify and characterize pregnancy-associated enzymes from maternal blood plasma.

Methods Employed: The principal methods employed are: (1) protein isolation techniques, including salt- and solvent-fractionation, gel-filtration, ion-exchange chromatography, and affinity chromatography; (2) enzyme assays, which are usually based on spectrophotometric or radiometric methods; (3) the zymogram technique, with which electrophoretic variants of enzymes can be detected; (4) organic-compound synthesis techniques, including ion exchange-, paper-, and thin-layer chromatography and gas chromatography-mass spectrometry.

Major Findings: (1) In a study of the control mechanism of gene expression in choriocarcinoma cells, we found that alkaline phosphatase in these cells can be induced by a variety of physiological and nonphysiological agents, such as dibutyryl cyclic AMP, 5-bromodeoxyuridine, and inhibitors of DNA synthesis (ara-C, mitomycin C, and phleomycin). The increase in alkaline phosphatase activity by these agents is indirect induction and requires both RNA synthesis and protein synthesis. Since acid phosphatase and phosphatase with a neutral pH optimum are not affected by the inducers, the induction is specific for alkaline phosphatase. The induced enzyme resembles the basal enzyme in its heat stability and kinetic properties. Furthermore, the induced enzyme and the basal enzyme have K_m values similar to that of alkaline phosphatase of term placenta. (2) Glycogen synthetase has been extensively purified from human placenta and from choriocarcinoma cells. The two enzymes were characterized with respect to their kinetic and regulatory properties. They are similar in many respects, but the choriocarcinoma enzyme has a K_a for glucose-6-P which is 5 to 10 times greater than the K_a of the placental enzyme. Also, whereas the placental D-form enzyme can be converted into I-form enzyme by incubating the partially purified enzyme (which contains synthetase-D phosphatase) in the presence of Mn^{++}, the D- to I-form conversion could not be demonstrated in preparations of synthetase from choriocarcinoma cells. (3) $MnSO_4$ has been found to be, like the sugar phosphates, an activator of D-form glycogen synthetase: $MnSO_4$ activates the enzyme in the absence of glucose-6-P, whereas Mn^{++} and SO_4^{--} separately only slightly effect the activity. On the other hand, Mn^{++} does activate the enzyme in the presence of glucose-6-P; Mn^{++}, unlike Mg^{++} and Ca^{++}, reduces the K_a for glucose-6-P to a value that is only one-tenth of the K_a in the absence of Mn^{++}. Under prevailing physiological concentrations of glucose-6-P and UDP-glucose, both $MnSO_4$ and Mn^{++}, at concentrations below 1 mM, significantly activate the enzyme. (4) In an effort to establish a function for placental alkaline phosphatase, an enzyme which is produced ectopically by some nontrophoblastic neoplasms, we found that this enzyme is a phosphoprotein phosphatase. It hydrolyzes phosphorylated histone and glycogen synthetase at a physiological pH. (5) Multiple forms of cyclic AMP-independent phosvitin kinases have been separated from an extract of rabbit muscle. These phosvitin kinases phosphorylate glycogen synthetase differently; also, they have different pH optima and have different requirements for Mg^{++} and for nucleotides as phosphoryl donors. (6) An enzyme designated α-ketoadipate reductase, which has not previously been described and which mediates the NADH-dependent reduction of α-ketoadipate, has been purified 1000-fold from human placenta, and many of its kinetic properties have been elucidated. An extract of placenta has also been shown to convert α-hydroxyadipate to a compound believed to be 1,2-butene-dicarboxylate. The properties of this enzyme, designated α-hydroxyadipate dehydratase, are currently under study. The discovery of these two enzymes in human placenta is the basis for the proposal of an alternate route of lysine degradation beginning with α-ketoadipate which is quite different from the currently accepted direct oxidative decarborylation of α-ketoadipate. A model, based on this new route, has been proposed which may help to explain the motor abnormalities in α-ketoadipic aciduria and glutaric aciduria, two metabolic defects of lysine metabolism which are accompanied by mental retardation.

Significance to Biomedical Research and the Program of the Institute: The investigation of placental enzymes in normal pregnancy will provide a suitable baseline with which to compare the same enzyme systems in placental explants, in benign and malignant trophoblast cells in culture, and in placental tissues from selected reproductive disorders.

The study of the control of gene expression of alkaline phosphatase is of importance because this enzyme is derepressed in many nontrophoblastic tumors. This frequent occurrence of derepression in tumors *in vivo*, considered together with the ease with which derepression can be achieved *in vitro*, suggests that the ectopic presence of the enzyme may serve as an early signal of malignant transformation. The development of a sensitive radioimmunoassay for this phosphatase would help us to elucidate the mechanism of enzyme induction by enabling us to detect catalytically nonactive forms of the enzyme. Also, because of increased sensitivity, the assay possibly could serve as a method for early detection of certain malignant transformations.

Investigation of the regulation of glycogen synthetase in the human placenta is essential for the understanding of fetal carbohydrate metabolism, which is greatly influenced by the placenta before the fetal liver has fully developed. Futhermore, by using glycogen synthetase as a model system, we learn more about phosphorylation and dephosphorylation reactions, key processes involved in the regulation of cellular metabolism, differentiation, and growth.

The elucidation of the lysine metabolic pathway is fundamental to the investigation of several recently discovered metabolic defects which relate to an inability to metabolize this amino acid. These disorders (hyperlysinemia, saccharopinuria, α-aminoadipic aciduria, α-ketoadipic aciduria, and glutaric aciduria) have been observed in association with mental retardation and motor abnormalities.

Proposed Course of Project: (1) Characterization of selected enzymes from placenta, maternal plasma, amniotic fluid, and trophoblastic cells in culture will continue. (2) Correlation between abnormal pregnancies and variations in fetal or maternal enzymes will be sought.

Publications:

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2. Fjellstedt, T. A. and Robinson, J. C.: Purification and properties of L-lysine-α-ketogluterate reductase from human placenta. Arch. Biochem. Biophys. 168: 536-548, 1975.

3. Fjellstedt, T. A. and Robinson, J. C.: Properties of partially purified saccharopine dehydrogenase from human placenta. Arch. Biochem. Biophys. 171: 191-196, 1975.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00087-04 LBS

PERIOD COVERED July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Development of Mammalian Metabolic Regulation: Glucose Homeostasis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. Glinsmann	Chief, Section on Physiological Controls	LBS	NICHD
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LAB/BRANCH

Laboratory of Biomedical Sciences

SECTION

Section on Physiological Controls

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	4.5	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

We have focused studies on galactose metabolism since it appears to have an important role in perinatal glucose homeostasis. In perinatal Rhesus liver, galactose markedly amplifies regulation of glucose production and glycogen synthesis in response to alterations in circulating glucose concentration.

Our recent experiments focus on: (1) effects of galactose on perinatal glucose and glycogen regulation in isolated liver; (2) the effects of glucocorticoids and glucagon on fetal rat liver galactose metabolism in vitro; (3) testing galactose in intravenous alimentation solutions for potential therapeutic benefit; (4) the glucoregulatory role of galactose in human polymorphonuclear leukocytes; and (5) the effects of maternal administration of β-methasone and dietary galactose on the development of fetal liver glycogen synthesis.

PHS-6040
(12-75)

Project Description:

Objectives: This project seeks to define in mammalian cells: (1) mechanisms underlying differential modulation of cellular metabolism by hormones and nutrients and (2) factors controlling development of these mechanisms. A principal focus is on factors which regulate glucose homeostasis during the newborn period. Companion studies and related biochemical mechanisms are reported in Project No. Z01 HD 00094-01 LBS.

Methods Employed: Isolated cell and organ cultures, isolated organ perfusions, and in vivo animal models are used for studying development of liver glucose production, glycogen and galactose metabolism, and concommitant regulation and developmental changes in rate-limiting enzymes. Potential therapeutic applications of these studies are examined in human neonates.

Major Findings: The activities of the rate-limiting enzymes of glycogen synthesis and degradation in liver are controlled by phosphorylation reactions. These reactions, which are important in minute to minute regulation of circulating glucose levels, are acutely altered by hormones and directly by glucose levels. We have recently found that galactose amplifies regulation of these enzymes by glucose. Thus, we have expanded our investigations into the properties and metabolism of galactose.

In isolated perinatal Rhesus livers, we confirmed and extended our observations that glucose regulation of glycogen cycle enzyme activities is minimal or absent and that galactose markedly facilitates this regulation. In addition, these livers release glucose faster in response to hypoglycemia when galactose is present in the circulation. This is also true of infants of streptozotocin-induced glucose-intolerant mothers.

We speculate that the differences in glucose regulation of liver glycogen metabolism may be contributory to hypoglycemia and the impairment of glucose tolerance which occurs in the newborn period and that galactose may have therapeutic applications in hyperalimentation therapy. In collaboration with Children's Hospital, we have established protocols to test this hypothesis. To date, we have nearly finished a description of normal human blood galactose levels in milk-feeding infants; intravenous alimentation protocols are beginning.

A collaborative study with the NPMB and the PRB has shown that fetal liver glycogen is greatly increased in fetuses of β-methasone-treated mother Rhesus monkeys. We have duplicated this effect in rats and correlated it with development of glycogen cycle enzymes. Galactose administered in maternal diet increases fetal liver glycogen near term when fetal glycogen cycle enzymes are developed.

The developmental time course of changes in galactose metabolism and galactose enzymes has been described in rats and soon will be completed in monkeys. Attempts to influence by hormones the time course of this development in an isolated rat liver organ-culture system have been unsuccessful; however, we have found that galactose-dependent glucose release is markedly influenced by glucocorticoids and glucagon. Initial studies indicate that this is related to changes in glucose-6-phosphatase activity.

The effect of galactose on glucose regulation is also present in human polymorphonuclear leukocytes. Although galactose is metabolized minimally in this tissue and by itself galactose does not alter glycogen synthesis, in its presence stimulation of glycogen synthetase activation and glycogen synthesis by glucose is markedly enhanced. This raises the possibility that galactose also may have glucoregulatory roles in tissues that do not employ it as a significant energy source.

In other experiments we found that galactose metabolism and galactose-metabolizing enzymes are markedly increased in animals made glucose-intolerant by administration of streptozotocin.

Significance to Biomedical Research and the Program of the Institute: Understanding mechanisms controlling the development of glucose homeostasis is important to our understanding of the metabolic abnormalities which occur in this area during perinatal life. In addition, the regulatory systems involved are ones which have been used to define basic metabolic controls by protein phosphorylation. Investigation of these systems deals with the important area of mechanisms controlling development of hormonal regulatory systems.

Proposed Course of Project: We plan to continue these projects as outlined and to expand studies to test whether increased galactose metabolism is a general feature of the diabetic state. Also, we intend to initiate studies on gluconeogenic regulation in the perinatal period.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00088-06 LBS

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Neuroendocrine Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. C. Klein	Physiologist	LBS	NICHD
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	M. Buda	Visiting Fellow	LBS	NICHD
	C. Y. Chiou	Guest Worker	LBS	NICHD
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B. Schrier and M. Mata, Behavioral Biology Branch, NICHD;
K. L. Kirk, Lab. of Chemistry, and T. Oka, Lab. of Biochem. and Met., NIAMDD.

LAB/BRANCH

Laboratory of Biomedical Sciences

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Section on Physiological Controls

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The molecular mechanisms controlling neuroendocrine processes are studied. Special emphasis is given to the adrenergic-adenosine 3',5'-monophosphate regulation of pineal N-acetyltransferase activity. This enzyme controls large changes in the production of the pineal product, melatonin. The effects of physiological variables on the development and function of this enzyme are investigated. These include environmental temperature, stress, and hormones. In addition, the effects of antidepressants and sympathomimetic drugs are studied. Another area of interest is circadian rhythms. The large circadian rhythm in pineal N-acetyltransferase is analyzed as to the factors and central neural structures which control this rhythm.

PHS-6040
(12-75)

Project Description:

Objectives: (1) To describe the molecular mechanism through which neuronal activity regulates biochemical activity in a neuroendocrine tissue, in the pineal gland, and in the brain; (2) to determine the developmental, central neural, environmental, and pharmacological factors influencing this mechanism; and (3) to study the nature of circadian rhythms.

Methods Employed: (1) To describe the molecular mechanism through which neuronal activity regulates biochemical activity in the pineal gland, rat pineal glands are cultured. This provides a well-defined and easily controlled experimental system. Cultures are treated with neurotransmitters, hormones, and drugs. The effects of these treatments are studied by measuring (a) the amount of cyclic AMP in the tissue; (b) the activity of N-acetyltransferase, the enzyme that converts serotonin to N-acetylserotonin and by doing so regulates the formation of melatonin; (c) the amount of serotonin in the gland; and (d) the activity of adenyl cyclase. The regulation of brain serotonin metabolism is studied in similar fashion using small sections of specific areas of brain.

Major Findings: (1) The administration of drugs which inhibit the uptake of catecholamines into nerve endings causes a much greater increase in pineal N-acetyltransferase activity in stressed animals as compared with the increase seen after stress alone or after injection of drug. This and other observations provide strong support of the concept that sympathetic nerve endings protect the pineal gland, and perhaps other sympathetically innervated tissues, from adrenergic stimulation by circulating catecholamines released during stress. (2) The regulation of pineal N-acetyltransferase activity is influenced by temperature. The response of cultured pineal glands to adrenergic stimulation is greater at a lower temperature (34 C) as compared with that at higher temperatures (37 or 40 C). A similar relationship is seen in neonatal animals *in vivo* when body temperature is changed by changing the environmental temperature. This observation suggests that the function of the pineal gland might be altered markedly by small alterations in body temperature. (3) The new amino acid analogue, 2-fluoro-L-histidine, blocks the induction of liver aryl hydrocarbon hydroxylase and liver tyrosine amino transferase in addition to pineal N-acetyltransferase. This indicates that the inhibitory effects of this compound are not unique to the pineal gland. 2-Fluoro-L-histidine should be a valuable tool to use in studying the biological function of histidine. (4) It was found that neither histamine or gamma amino butyric acid, both of which are found in the pineal gland and are transmitters in other tissues, can influence the adrenergic-cyclic AMP regulation of pineal N-acetyltransferase. This provides more evidence of the independence of this regulatory system. (5) Substantial differences in the regulatory system controlling pineal N-acetyltransferase activity in rat and chickens have been observed. Whereas this enzyme increases at night in both species, this increase is mediated by the superior cervical ganglia in the rat but not the chicken. Whereas the activity decreases rapidly in both species following exposure to light, only in the rat is this dependent upon intact eyes. Whereas the activity of N-acetyltransferase is increased by isoproterenol in the rat, similar injections to chickens have no effect on enzyme activity. (6) Pineal N-acetyltransferase activity can be stimulated in monkey pineal glands by treatment with adrenergic drugs. Although this effect is much smaller and more variable than in the rat, it would appear that a similar regulatory system exists in both the rat and monkey. (7) Rat pineal cells can be cultured in monolayer. These cells contain N-acetyltransferase activity which is regulated by a mechanism which possesses the same features as that described for rat pineal glands using organ culture techniques.

Significance to Biomedical Research and the Program of the Institute: (1) The pineal gland can control the growth and activity of reproductive organs. The increased understanding of the factors which regulate this gland will increase our ability to control it and to understand how it functions under a wide spectrum of physiologically significant circumstances. (2) Our understanding of the effects of 2-fluoro-L-histidine will allow us to increase our knowledge of the function of histidine in proteins, primarily enzymes and histones. (3) The development of a monolayer cell system for the culture of pineal cells will provide a means to study both biochemistry and histochemistry of these cells, and thus provide a more adequate description of the structure and function of the pineal gland. The cell method should lead to the development of means to grow large numbers of pineal cells, allowing for the large scale production and harvesting of pineal products and pineal enzymes. These will be valuable tools to use in extending our studies of the pineal gland. In addition, pineal products represent the prototypes of drugs used to control reproductive function.

Proposed Course of Project: (1) Investigation of the regulation of the production of melatonin will continue with emphasis given to the molecular mechanisms involved in the regulation of pineal N-acetyltransferase activity. Areas to be included are sulfur-containing amino acids and their derivatives, cyclic AMP, endogenous inhibitors of adenyl cyclase, and phosphorylation reactions. (2) Studies on the mode of action of 2-fluoro-L-histidine as an inhibitor of enzyme induction will continue, with the primary purpose of these studies being to determine if 2-fluoro-L-histidine is incorporated into a specific enzyme, tyrosine aminotransferase. (3) Studies on ornithine decarboxylase will concentrate on the relation of the activity of this enzyme to the activity of pineal N-acetyltransferase. (4) Investigation of the nature of a non-arginine vasotocin pineal peptide will continue; purification and development of an immunoassay will be the goals. (5) Studies on the pinealocyte cell culture will continue with the primary goals being to enrich the cultures with pinealocytes, to obtain a transformed cell line, and to establish cell cultures of monkey and sheep pineal cells. (6) Studies on the central nervous system structures involved in the regulation of the pineal gland will continue with the main efforts devoted to describing the structures regulating N-acetyltransferase and other pineal enzymes. (7) New projects will include studies on the effects of drugs on the development of the system which regulates pineal N-acetyltransferases, the description of melatonin in human serum and urine, the study of several circadian rhythms and their control by the suprachiasmatic nucleus, and the study of the biological effects and half-lives of 6-fluoromelatonin and 4,6-difluoromelatonin.

Publications:

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2. Klein, D. C. and Parfitt, A.: Ouabain blocks the adrenergic-cyclic AMP stimulation of pineal N-acetyltransferase activity. In Weiss, B. (Ed.): Cyclic AMP in Disease. Baltimore, University Park Press, 1975, pp. 251-265.

3. Binkley, S., Klein, D. C. and Weller, J. L.: Pineal serotonin N-acetyltransferase activity: Protection of stimulated activity by acetyl CoA and related compounds. J. Neurochem. 26: 51-55, 1976.

4. Parfitt, A., Weller, J. L. and Klein, D. C.: Beta adrenergic-blockers decrease adrenergically stimulated N-acetyltransferase activity in pineal glands in organ culture. Neuropharmacology, in press.

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8. Mata, M., Schrier, B. and Klein, D. C.: Studies on γ-Amino butyric acid in the pineal gland. Brain Res., in press.

9. Chen, C., Klein, D. C. and Robinson, J. C.: Monoamine oxidase activity in rat placenta, human placenta and choriocarcinoma cells in culture. J. Reprod. Fertil. 46: 477-479, 1976.

10. Ralph, C. L., Binkley, S., MacBride, S. E. and Klein D. C.: Regulation of pineal rhythms in chickens: Effects of blinding, constant light, constant dark, and superior cervical ganglionectomy. Endocrinology 97: 1373-1378, 1975.

11. Klein, D. C. and Kirk, K. L.: 2-Fluoro-L-Histidine: A histidine analog which inhibits enzyme induction. Symposium on Biochemistry Involving the Carbon-Fluoride Bond. ACS Symposium Series, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00089-02 LBS

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Pineal-pituitary Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. C. Klein	Physiologist	LBS	NICHD
OTHER:	J. M. Martin	Research Chemist	LBS	NICHD

COOPERATING UNITS (if any)

B. Weintraub, CEB, NIAMDD; E. Gross, RRB, NICHD; K. L. Kirk, LC, NIAMDD.

LAB/BRANCH
Laboratory of Biomedical Sciences

SECTION
Section on Physiological Controls

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of the pineal gland on pituitary gland function are investigated by determining the effects of pineal products on the LH-response of neonatal rat pituitary glands to treatment with LH-releasing factor. It has been discovered that melatonin can block this response and that it acts rapidly through a relatively specific mechanism at low concentrations. The effects of melatonin have been observed in organ culture and in vivo. Efforts to synthesize a long acting melatonin analog have produced 4,6-difluoromelatonin and 6-difluoromelatonin. These compounds were found to be as potent as or more potent than melatonin. In addition to melatonin, other pineal products including serotonin, methoxytryptamine, and pineal peptides were found to be active in blocking the effects of LRF on the pituitary gland.

Project Description:

Objectives: To chemically and physiologically characterize pineal products which influence reproductive function.

Methods Employed: A pituitary organ culture system which uses neonatal rat pituitary glands is used. This method provides a highly sensitive and precise assay system for the measurement of the effects of pineal hormones on the pituitary gland. Pineal extracts, extracts of pineal culture medium, and chemicals are tested in this system. Classical methods of extraction and purification are used to make test preparations. The preparations are tested for their ability to suppress the release of LH from the pituitary gland treated with LH-releasing factor, and from the untreated pituitary gland. In conjunction with the in vitro studies, in vivo experiments are done. In these experiments animals are injected with LH-releasing factor and a test compound; serum LH is measured.

Major Findings: (1) An active principal in pineal culture medium was suspected to be melatonin. This led to experiments in which melatonin was found to be a potent and rapid inhibitor of LH-releasing factor-stimulation of the release of LH. Other studies indicated that N-acetylserotonin, hydroxyindoleacetic acid, hydroxytryptophol, methoxytryptophol, and methoxyindoleacetic acid were ineffective. Serotonin was active at low concentrations but did not inhibit LH release more than 50%; methoxytryptamine was as effective as melatonin at 100-1000-fold higher concentrations in completely blocking the release of LH from glands treated with LH-releasing factor. (2) Studies in vivo showed that melatonin was also effective in blocking the LH-releasing factor-induced release of LH. (3) Further analysis of pineal culture medium confirmed the belief that melatonin was an active principal; evidence was also accumulated which indicated that serotonin was also an active principal inhibiting the release of LH. (4) Two derivatives of melatonin, 6-fluoromelatonin and 4,6-difluoromelatonin, were found to be as potent as or more potent than melatonin in the in vitro test system.

Significance to Biomedical Research and the Program of the Institute: The finding that melatonin is a potent inhibitor of the release of LH raises the possibility that this compound might be used as a drug. The finding that the fluoroderivatives of melatonin are also active may lead to longer acting drugs which are resistent to enzymatic degradation.

Proposed Course of Project: (1) The identity of pineal peptides will be pursued. Studies on the mechanism of action and specificity of melatonin as an inhibitor of the LH-releasing factor-stimulated release of LH will be continued. The immediate goal will be to develop a melatonin derivative which is more potent than melatonin. (2) The effects of arginine vasotocin on reproductive function will be determined on a collaborative basis.

Publications:

1. Martin, J. E. and Klein, D. C.: Melatonin: Inhibition of luteinizing hormone releasing factor-induced LH release. Science 191: 301-302, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00090-04 LBS

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The Effect of Metabolic Disorders on the Biochemistry of the Developing Brain. (PREVIOUS TITLE: The Effect of Metabolic Disorders on the Biochemistry, Structure, and Function of the Developing Brain)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Guroff	Chief, Sec. on Intermediary Metabolism	LBS NICHD
OTHER:	M. Weston	Biologist	LBS NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biomedical Sciences

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 0.25	PROFESSIONAL: 0.16	OTHER: 0.09

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this research are to produce biochemical models of mental retardation syndromes in laboratory animals by chemical means. By a study of the mechanisms by which specific chemical alterations produce changes in normal neural functioning, it is hoped that new methods for preventing such alterations may be found, thus pointing the way for new clinical approaches. Recently, it has been shown that treatment of newborn rats with p-chlorophenylalanine plus phenylalanine produces a biochemical picture similar to that seen in clinical phenylketonuria and that such treatment during the development of the rat produces, in the adult animal, behavioral and pathological changes similar to those seen in adult human beings suffering from phenylketonuria. Current studies are directed toward: (1) discovering a treatment which will prevent or reverse the behavioral changes seen in the model; (2) exploring the use of other, less toxic inhibitors; (3) pinpointing the exact developmental period of the insult.

Project Description:

Objectives: The objectives of this research are: (1) to produce biochemical models of mental retardation syndromes in laboratory animals by chemical means; (2) to investigate the pathological and behavioral aspects of the chemical treatments; (3) to study the mechanisms by which specific chemical alterations produce a change in normal neural functioning; (4) to attempt new methods for preventing such alterations, hopefully pointing the way to new clinical approaches.

Methods Employed: The methods employed include standard enzymatic assay techniques, histological examinations, and behavioral evaluation.

Major Findings: It is necessary to treat newborn animals with p-chlorophenylalanine plus phenylalanine for at least 21 days to produce the behavioral change.

Significance to Biomedical Research and the Program of the Institute: In general, it has been impossible to study the etiology of mental retardation in the laboratory because of the lack of an adequate model. The model available from the work in this laboratory is the best characterized in both biochemical and behavioral aspects. Phenylketonuria is an appropriate disease to study in this respect because a great deal is known about the enzyme which is deleted, phenylalanine hydroxylase, and about the biochemical and behavioral consequences of such deletion in human beings. Such a model should provide a tool with which to study both the causes and the possible amelioration of this disease in children and adults.

Proposed Course of the Project: If we decide to continue on this project at the present time, we will pursue one of the following lines: (1) attempt to verify a recent report that 5-hydroxytryptophan administration will prevent the damage done by p-chlorophenylalanine plus phenylalanine; (2) inspect the biochemical and behavioral effects of α-methylphenylalanine, which has recently been reported to produce an irreversible inhibition of the phenylalanine hydroxylase, as does p-chlorophenylalanine, with no substantial mortality; (3) proceed to give larger doses of the p-chlorophenylalanine plus phenylalanine regimen for short periods to the preweaning rat hoping to find a vulnerable period.

Publications:

1. Rowe, V.D., Fales, H.M., Pisano, J.J., Andersen, A.E. and Guroff, G.: Urinary metabolites of phenylalanine in the preweanling rat treated with parachlorphenylalanine and phenylalanine. Biochem. Med. 12: 123-136, 1975.

2. Guroff, G.: Some aspects of aromatic amino acid metabolism in the brain, 1975. In Tower, D. B. (Ed.): The Nervous System. NINDS 25th Anniversary Volume, The Basic Neurosciences. New York, Raven Press, 1975, Vol. I, pp. 553-564.

3. Andersen, A.: Maternal hyperphenylalaninemia: An experimental method in rats. Dev. Psychobiol., in press.

4. Andersen, A. and Avins, L.: Brain phenylalanine in experimental phenylketonuria: Lowering by competition with other neutral amino acids for a common transport system. Arch. Neurol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00091-04 LBS

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mechanisms of Aromatic Hydroxylation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Guroff	Chief, Sec. on Intermediary Metabolism	LBS	NICHD
OTHER:	C. Letendre	Research Chemist	LBS	NICHD
	T. Kato	Visiting Fellow	LBS	NICHD
	K. Nagaiah	Visiting Fellow	LBS	NICHD

COOPERATING UNITS (if any)
H. Kon, LCP, NIAMDD; J. Daly and D. Jerina, LC, NIAMDD; T. Shiota, U. of Alabama

LAB/BRANCH
Laboratory of Biomedical Sciences

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.17	2.17	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this research are to provide general information on the process of enzymatic hydroxylation and, more specifically, on the characteristics of phenylalanine hydroxylase and its pteridine cofactor. Recently, the phenylalanine hydroxylase from Pseudomonas sp. has been partially purified and its mechanisms of activation and action investigated. It has been shown to be a small, pteridine-requiring hydroxylase with a stringent iron requirement. Current efforts are directed toward: (1) purifying the enzyme to homogeneity via affinity chromatography on pteridine-agarose; (2) investigating the state of the iron in the molecule; (3) preparing and characterizing antibody to the enzyme; (4) isolating the mRNA for the enzyme.

Project Description:

Objectives: The objectives of this research are: (1) to provide information on the process of enzymatic hydroxylation; (2) to get detailed information on the nature of phenylalanine hydroxylase; (3) to purify and characterize accessory enzymes involved in hydroxylation; (4) to investigate relationships between pteridine reductase, pteridine biosynthetic enzymes, and the hydroxylase itself; (5) to provide information on the enzymes in the biosynthetic pathway to the pteridine ring; and (6) to investigate the functions of pteridines in addition to their actions as hydroxylase cofactors.

Methods Employed: The methods employed include standard techniques in enzyme kinetics and purification, antibody detection, organic synthesis and characterization, isotope quantitation, high voltage electrophoresis, and fluorescence measurements. Also used are methods of protein characterization including molecular weight determinations and measures of purity, and physical methods such as e.s.r. for investigating the nature of enzyme proteins.

Major Findings: (1) Antibody to bacterial phenylalanine hydroxylase does not cross-react with the comparable liver enzyme; (2) the products of tyrosine metabolism inhibit the liver phenylalanine hydroxylase, as well as the bacterial enzyme; (3) pteridine-agarose is a useful adjunct to the purification of the enzyme.

Significance to Biomedical Research and the Program of the Institute: Information about phenylalanine hydroxylase and other hydroxylases and about ways in which to inhibit or stimulate them relates directly to the general problems of normal neural function and of mental retardation. Phenylalanine hydroxylase is the missing enzyme in the best characterized of the genetic retardations, phenylketonuria. Detailed analysis of the structure and mechanism of this enzyme should provide some information on the nature of hydroxylation and on methods by which to manipulate the activity of this important system. Further, it should be possible, upon examining the detailed chemistry of the pteridine reductase associated with the hydroxylase, to understand the relationship between the proteins which carry the activities of these enzymes. Finally, since the pteridine cofactors are important in a number of hydroxylase reactions including those leading to the neurohormones serotonin and norepinephrine, information about the pharmacology and biochemistry of the pteridine biosynthetic pathway may lead to the ability to inhibit or stimulate these pathways, and perhaps to new therapeutic tools for treating maladies involving these neurohormones.

Proposed Course of the Project: (1) Continued study of methods for the purification of the enzyme; (2) investigation of the iron component of the enzyme; (3) use of the antibody to attempt the isolation of the messenger RNA for the enzyme.

Publications:

Letendre, C. H., Dickens, G., and Guroff, G.: Phenylalanine hydroxylase from *Pseudomonas* sp. (ATCC 11299a). Purification, molecular weight, and influence of tyrosine metabolites on activation and hydroxylation. *J. Biol. Chem.* 250: 6672-6678, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00092-04 LBS

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biochemistry of Brain Macromolecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Guroff	Chief, Sec. on Intermediary Metabolism	LBS NICHD
OTHER:	P. MacDonnell	Staff Fellow	LBS NICHD
	N. Tolson	Biologist	LBS NICHD
	M. Weston	Biologist	LBS NICHD

COOPERATING UNITS (if any)
W. Lovenberg and P. Lerner, HE, NHLI; K. Catt and R. Rebar, RRB, NICHD; and T. Lloyd, U. of Pennsylvania.

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Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 2.5	PROFESSIONAL: 0.67	OTHER: 1.83

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this research are to investigate the translation and regulation of macromolecules of neural interest. Attempts are being made to localize and translate the messenger RNA for the enzyme tyrosine hydroxylase. If such a message can be measured, it will be used as a tool for the study of the induction of the enzyme by nerve growth factor, and for a number of experiments on other influences which impinge on the enzyme. Other experiments are pointed at the isolation of the message for the nerve growth factor itself. Another line of investigation involves a search for the mechanism of the biosynthesis of the proteolipid proteins which are found in the myelin. Earlier data from other laboratories indicated that these proteins were made in the mitochondria. It has been shown recently that mitochondria do, indeed, make proteins which have the general characteristics of the proteolipids, and that the mitochondria from young brain make much more than do comparable mitochondria from the brains of adults or from the livers of newborn animals. If it can be shown that the synthesized material is indeed the proteolipid of the myelin, then an attempt will be made to isolate, translate, and characterize the messenger RNA.

Project Description:

Objectives: The objectives of this research are: (1) to investigate the synthesis and function of brain macromolecules; (2) to compare, characterize, and identify the products made in a cell-free system using different populations of brain mRNAs; (3) to translate specific mRNAs for proteins of neurochemical interest and study their regulation and development.

Methods Employed: The methods include those used to isolate and characterize messenger RNAs; that is, extraction, centrifugation, hybridization to oligo(dT)-cellulose, gel electrophoresis, cell-free protein synthesis using a system from wheat germ. In addition, methods involving the production and use of specific antibodies are necessary.

Major Findings: None

Significance to Biomedical Research and the Program of the Institute: The translation of specific messengers of neural interest into their protein products, and the assay of the levels of these messengers under various conditions, can increase our understanding of normal neuronal function. The studies of tyrosine hydroxylase message give promise of being able to assay this important messenger under a variety of physiological conditions, including those which increase sympathetic activity, such as stress. The nerve growth factor message can be used, hopefully, to study the development and the function of this important regulatory substance. Newer studies on the translation of proteolipid message may help to understand the nature and development of the process of myelination. An understanding of the synthesis and function of these RNAs may provide tools for the investigation of the biochemical alterations occurring in various neural disorders.

Proposed Course of the Project: (1) Further attempts to translate the messenger RNA for tyrosine hydroxylase, or for another of the enzymes induced by the nerve growth factor; (2) further work on the synthesis of nerve growth factor from its mRNA; (3) characterization of the chloroform-methanol soluble products produced by mitochondria from immature brain, and possible identification of the myelin proteolipid among them.

Publications:

1. DeLarco, J., Nakagawa, S., Abramowitz, A., Bromwell, K. and Guroff, G.: Polyadenylic acid-containing RNA from rat brain synaptosomes. J. Neurochem. 25, 131-137, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00093-02 LBS

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Mechanism of Action of Nerve Growth Factor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Guroff	Chief, Section on Intermediary Metabolism	LBS	NICHD
OTHER:	V. Rowe	Research Associate	LBS	NICHD
	L. Avins	Research Associate	LBS	NICHD
	B. Nikodijevic	Visiting Scientist	LBS	NICHD
	M.-Y. Yu	Senior Staff Fellow	LBS	NICHD
	P. MacDonnell	Staff Fellow	LBS	NICHD
	J. Lakshmanan	Visiting Fellow	LBS	NICHD

COOPERATING UNITS (if any)
H. Pollard and O. Nikodijevic, RRB, NICHD; B. Schrier, BB, NICHD; D. Klein, LBS, NICHD; R. Eldridge, IR, NINCDS; and T. Lloyd, U. of Pennsylvania.

LAB/BRANCH
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SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this research are to provide information on the mode of action of nerve growth factor. Nerve growth factor is required for the development and maintenance of the sympathetic nervous system and may be prototypic for factors involved in differentiation of various cell types. The mechanism by which nerve growth factor induces the enzymes involved in the synthesis of the sympathetic transmitter, norepinephrine, must be understood to permit a complete understanding of the mechanism. Finally, nerve growth factor action must be understood before complete information is available about the development and differentiation of the non-dividing, non-replaceable neurons of the central nervous system.

Project Description:

Objectives: (1) To investigate the nature of the action of nerve growth factor on the growth and maintenance of the sympathetic nervous system; (2) to understand the general implications of the action of the nerve growth factor on these cells and to determine if there are other factors which direct the development of other types of neurons; (3) to search for possible sites of action of the nerve growth factor in the central nervous system; (4) to measure the nerve growth factor in clinically relevant conditions, such as those involving tumors of the sympathetic nervous system or of other possible nerve growth factor-responsive tissues.

Methods Employed: The methods include standard techniques of cell culture and *in vivo* investigations on the sympathetic nervous system. Enzyme assay and small animal surgery are used, as well as biological methods by which nerve growth factor action is measured. Standard antibody techniques are employed for the preparation and implementation of the relevant antibodies.

Major Findings: (1) Under the appropriate conditions the level of tyrosine hydroxylase in sympathetic ganglia can be maintained and even increased *in vitro* by nerve growth factor; (2) the addition of nerve growth factor to such cultures results in an increase in the concentration of cyclic AMP in the ganglia; (3) the addition of nerve growth factor to isolated adrenal chromaffin granule membranes results in a change in the cyclic AMP concentration, in this case, a decrease; (4) the addition of dibutyryl cyclic AMP and theophylline results in an increase in tyrosine hydroxylase, thus mimicking the effect of nerve growth factor; (5) the nerve growth factor antibody can be purified to homogeneity using affinity columns; (6) the synthesis of tyrosine hydroxylase in the ganglia in culture can be measured by a combination of isotopic and immunological methods; (7) pineal cells can be grown in dissociated cell cultures and will support the growth of sympathetic neurons in the absence of added nerve growth factor; (8) the ganglia in culture can be used as the basis of a quantitative assay for nerve growth factor and may have value as a clinical measure.

Significance to Biomedical Research and the Program of the Institute: Nerve growth factor is the best characterized and, indeed, the first known factor which influences the growth and maintenance of sympathetic neurons. Its mechanism of action, therefore, is of extreme interest and is as yet unknown. Understanding of the mechanism of action of nerve growth factor might lead to a better understanding of the mode of regulation of the growth of neurons and of their differentiation into non-dividing, non-replaceable cells. Further, understanding of the mechanism by which nerve growth factor affects cell development may give clues as to the normal regulation in mammalian cells and, specifically in neurons, as to the process of genetic expression. Finally, studies on nerve growth factor may indicate ways in which targeting and directing of neural connectivity can be understood and, indeed, altered.

Proposed Course of the Project: (1) Continued study of the relation of nerve growth factor action to the levels of cyclic nucleotides in the cells; (2) characterization of the nerve growth factor mRNA (see Project No. Z01 HD 00092-04 LBS); (3) further investigation of the relationship between the receptor for nerve growth factor and the β-adrenergic receptor; (4) use of the tyrosine hydroxylase antibody to elucidate the molecular mechanism of the induction of tyrosine hydroxylase by the nerve growth factor *in vitro* and *in vivo*; (5) use of the purified antibody to determine the nature of the principle in pineal cells which is capable of supporting the growth of sympathetic neurons in culture; (6) use of the purified antibody to investigate the possible requirement for the presence of or synthesis of nerve growth factor in cultures of noradrenergic portions of the central nervous system; (7) use of the purified antibody to locate the site of nerve growth factor binding to the cells *in vitro* and to elucidate the mechanism of immunosympathectomy; (8) investigation of enzymes which degrade NGF, either by proteolysis or GSH disulfide exchange; (9) assay of sera from patients with relevant neural tumors for excess or absence of nerve growth factor.

Publications:

1. Nikodijevic, B., Nikodijevic, O., Yu, M.-Y., Pollard, H., and Guroff, G.: The effect of nerve growth factor on cyclic AMP levels in superior cervical ganglia of the rat. *Proc. Nat. Acad. Sci. U.S.A.* 72: 4769-4771, 1975.

2. Nikodijevic, O., Nikodijevic, B., Zinder, O., Yu, M.-Y., Guroff, G., and Pollard, H.: Control of adenylate cyclase from secretory vesicle membranes by β-adrenergic agents and nerve growth factor. *Proc. Nat. Acad. Sci. U.S.A.*, in press.

3. Stoeckel, K., Gagnon, C., Guroff, G., and Thoenen, H.: Purification of nerve growth factor (NGF) antibodies by affinity chromatography. *J. Neurochem.*, in press.

4. Stoeckel, K., Paravaccini, U., Guroff, G., and Thoenen, H.: The significance of retrograde axonal transport for the accumulation of systemically administered nerve growth factor in the rat superior cervical ganglion. *Brain Res.*, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00094-01 LBS

PERIOD COVERED July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Development of Mammalian Metabolic Regulation: Molecular Aspects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	W. Glinsmann	Chief, Section on Physiological Controls	LBS	NICHD
	H. J. Eisen	Medical Officer	LBS	NICHD
OTHER:	F. L. Huang	Senior Staff Fellow	LBS	NICHD
	C. Nakai	Visiting Fellow	LBS	NICHD

COOPERATING UNITS (if any)

K.-P. Huang, E. Itarte, and J. C. Robinson, Section on Developmental Enzymology, LBS, NICHD

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Laboratory of Biomedical Sciences

SECTION

Section on Physiological Controls

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 3.5	PROFESSIONAL: 3.5	OTHER: 0

SUMMARY OF WORK (200 words or less - underline keywords)

This project examines molecular mechanisms by which hormones and nutrients regulate metabolism. Current work focuses on (1) control of phosphotransferase and phosphatase reactions by cyclic AMP and polyamines and (2) purification of the glucocorticoid receptor.

PHS-6040
(12-75)

Project Description:

Objectives: This project seeks to define in mammalian cells: (1) mechanisms underlying differential modulation of metabolism by hormones and nutrients and (2) factors controlling development of these mechanisms. A general model is being examined based on protein phosphorylations regulating rate-limiting enzymes. Current interest centers on a comprehensive description of glycogen cycle enzymes, possible regulation of phosphotransfer reactions by naturally occurring polyamines, and purification of the glucocorticoid-receptor. Companion in vivo and in vitro studies are reported in Project No. Z01 HD 00087-04 LBS.

Methods Employed: Enzymes and regulatory proteins are isolated from rabbit and rat skeletal muscle and liver and from bovine heart tissues; their mode of regulation is studied.

Major Findings: A generally accepted mode of action of cyclic AMP involves activation of a protein kinase. Increasing evidence from our and other laboratories supports the contention that glycogen cycle enzymes, which are substrates for this kinase, have multiple phosphorylation sites, some of which are phosphorylated by other kinases. In collaboration with Drs. K.-P. Huang, E. Itarte, and J. C. Robinson, separation and partial purification of two kinases which phosphorylate rabbit muscle glycogen synthetase have been obtained; kinase requirements for nucleotides and divalent cations have been described (see Project No. Z01 HD 00086-06 LBS). The multiple phosphorylation sites appear to serve two functions; control of glycogen enzyme activities, and regulation of the ability of the enzymes to serve as substrate for subsequent kinase and phosphatase attack. Thus, the multiple phosphorylation sites are probably important in modulating the effects of the cyclic AMP-dependent kinase and hence hormonal effects mediated by cyclic AMP.

Little is known about the phosphoprotein phosphatases reversing the effects of cyclic AMP-dependent and other kinases which act at the multiple phosphorylation sites described above. Increasing evidence supports that nutrient- and insulin-mediated modification of the effects of cyclic AMP may be mediated via these phosphatases. Purification of these phosphoprotein phosphatases has been difficult to obtain because of multiple molecular species of the phosphatases and marked activation and apparent changes in substrate specificities that occur with purification. We found that this may be due to the presence of regulatory inhibitor proteins which dissociate from the enzyme(s).

Two relatively heat-stable phosphatase inhibitor proteins have now been separated, partially purified, and characterized. One has been shown to be a phosphoprotein which is phosphorylated by the cyclic AMP-dependent kinase and which in the phospho-form is a potent inhibitor of phosphorylase phosphatase. From studies with multiple phosphoprotein substrates, we have developed the hypothesis that these inhibitors function in phosphatase substrate recognition. Not only do the inhibitors appear to be selective for different protein substrates, but their effects on a given substrate appear to be modified by metabolic intermediates which bind to that substrate. Verification of this important possibility is being pursued with more purified preparations of phosphatase and inhibitors; evidence is being sought for physiological regulation of inhibitor activity and/or association of the inhibitors with specific phosphatases.

In mammalian cells, polyamine concentrations are markedly increased during periods of rapid growth; the naturally occurring polyamines (putrescene, spermidine, and spermine) stimulate DNA, RNA, and protein synthesis. We speculated that these polyamines may be acting by regulating phosphotransferases or phosphatases which are thought to be important in these reactions. Although to date we have been unable to detect any effect of these polyamines on phosphorylation or dephosphorylation of histones, phosphorylase-phosphatase activity is selectively inhibited by spermine.

Of potentially major importance is our observation that polyamines profoundly stimulate liver nucleosidediphosphate kinase. The effects of each polyamine are relatively selective for specific nucleosidediphosphates. Nucleosidediphosphate kinase is responsible for replenishing cells with various nucleosidetriphosphates which are required for the early stages of polysaccharide, lipid, and protein synthesis and which are the building blocks of nucleic acids. Therefore, the action of polyamines at the level of this enzyme may be related to their function in rapid tissue growth.

Glucocorticoids profoundly regulate the development of many enzyme systems; they augment cyclic AMP-mediated processes by as yet unknown mechanisms. The glucocorticoid receptor is therefore being purified for studies on its role in the control of gene expression and enzyme-activity regulation. A rapid two stage DNA-cellulose chromatography method has been developed which yields an 1800-fold purification of this receptor from rat liver.

Significance to Biomedical Research and the Program of the Institute. Regulation of cellular metabolism, growth, and differentiation is thought to be mediated by factors which alter cyclic AMP metabolism and the effects of cyclic AMP. Thus, understanding how the multiple effects of cyclic AMP may be differentiated relative to one another is important to our understanding of how cell metabolism can be differentially regulated.

The potential effects of polyamines in normal and neoplastic growth have been well described. Description of a role for these polyamines in controlling protein and nucleotide phosphorylations may offer important insight into their relationship to cell growth and metabolic regulation.

The details of how the glucocorticoid receptor functions are basically important to control of gene expression.

Proposed Course of Project: Characterization of enzymes participating in glycogen cycle phosphorylations will continue with particular emphasis being placed on the regulatory roles of multiple phosphorylation sites, newly described protein kinases, regulatory subunits of phosphoprotein phosphatases, and allosteric effectors of protein kinase and phosphatase reactions. We shall continue investigating a potential role of polyamines in regulating kinase and phosphatase reactions. We shall continue to purify the glucocorticoid receptor for studies on gene expression.

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ANNUAL REPORT
# of the
Reproduction Research Branch
National Institute of Child Health and Human Development
July 1, 1975 through June 30, 1976

SUMMARY

NICHHD's intramural population research activities are directed toward generating the scientific information necessary for development of methods for reversible, voluntary fertility control, and for diagnosis and rational treatment of human gonadal dysfunctions and endocrine diseases. These research activities are conducted by scientists in the Reproduction Research Branch, who are expert in the physical, chemical, biological, immunological, endocrinological and clinical skills required for these tasks. The scope of investigations conducted by these scientists ranges from analysis of physical, chemical and antigenic properties of purified gonadotropic hormones through studies of the physical and biochemical correlates of hormonal interactions with subcellular organelles and living cells isolated from ovary, testis and adrenal, to studies of the complex interactions between hypothalamus, pituitary, gonads, and genitalia in the intact animal.

The "cutting edge" of investigations in this area is currently at the molecular level. Thus, it can be shown that hormonal action is initiated by the binding of the hormone molecules to receptors, some in cell membranes and some in cell sap. This receptor-hormone interaction initiates the sequence of biochemical events which culminates in the characteristic "activities" of hormones upon the gonadal and other tissues which respond to these substances.

For the successful accomplishment of studies at the molecular level, much research must be directed toward developing new methods for studying the physical and biochemical basis of interactions between hormones and receptors. In the development of new methods it is not always possible to begin with the tissue or substance of ultimate interest by virtue of constraints imposed by limited quantities of suitable tissue or of purified chemicals. For example, the application of techniques such as nuclear magnetic resonance currently requires relatively large quantities of material, which are rarely available. Bacterial model systems have been used to develop methods for exploiting the potential for using stable isotopes in elucidating the physical and structural changes that occur during the hormone-receptor interactions involved in reproductive processes.

High resolution nuclear magnetic resonance, suitable for monitoring spectral behavior of ^{13}C at natural abundance, has been used to determine the structural basis of antigenic similarities of cell wall polysaccharides of strains of E. Coli and meningococcus group C polysaccharides. The coliform polysaccharide was shown to represent a hybrid of $\alpha 2,8$ and $\alpha 2,9$-linked heteropolymers of sialic acid whereas meningococcus group C polysaccharide consists of a pure $\alpha 2,9$-linked polymer of sialic acid. In addition, both ^{1}H and ^{13}C NMR were used to study the conformational surfaces of thiotepa, an agent useful in the treatment of human ovarian cancers. To increase the sensitivity of the NMR method, ^{13}C

amino acids are being used to synthesize peptides with known biological activities for further studies. It is expected that this technology can be applied to studies such as the conformation of the carboxy terminal peptide in human chorionic gonadotropin and the recently demonstrated similar hormone in pituitary extracts.

For meaningful studies of the molecular basis of hormonal action, purified substances are a necessary prerequisite. In addition, isolation and purification of natural substances is a necessary prelude to determination of amino acid sequence, and subsequently for the synthesis of biologically active proteins. Research and development in this area has consisted of optimization of the conditions for fractionating mixtures of proteins by developing computer based models for analysis of data from polyacrylamide gel electrophoresis, with and without SDS, for isoelectric focusing, and for gel filtration. This technology has been adapted to the fractionation of isohormones of human growth hormone with enhanced biological activity which are produced by enzymatic digestion of human growth hormone in pituitary extracts, and to the isolation of six milligrams of human prolactin from ten liters of human amniotic fluid. The modified growth hormone was shown to have an increased plasma half-life in human subjects, possibly accounting for its enhanced biological activity. The purified prolactin will be used to determine the amino acid sequence of the hormone.

The value of highly purified proteins and knowledge of their primary structure for advancing knowledge of the molecular basis of hormonal actions has been very clearly demonstrated in studies completed this year relating to human chorionic gonadotropin. These observations have the potential for being the most important development in the field of gonadotropin physiology since the discovery of human chorionic gonadotropin by Aascheim and Zondek in 1927. The amino acid sequence has been determined for a peptide isolated following tryptic digestion of the S-carboxy methylated desialated beta subunit of human chorionic gonadotropin. This peptide contains the 23 carboxy terminal amino acid residues of this hormonal subunit, and the sequence is not duplicated in any of the other human glycoprotein hormones (hLH, hTSH, or hFSH). In research done in collaboration with Dr. R. E. Canfield of Columbia University, this peptide was used to produce antisera in rabbits. To test the specificity of the antisera, synthetic peptides, containing varying numbers of amino acids in the sequence proposed by Canfield for this tryptic peptide, were used to inhibit the binding of ^{125}I-labeled hCG to these antisera in radioimmunoassays. It was shown that the last 18 amino acid residues of this peptide were involved in the antigenic site. This antiserum has been used to show that human pituitary extracts and extracts of urine from postmenopausal women, patients with primary diseases such as Klinefelter's Syndrome, and from normal men and women contained a substance with the physical antigenic and biological properties of human chorionic gonadotropin. These observations have far-reaching implications for development of suitable pregnancy tests, for the diagnosis of tumors secreting chorionic gonadotropin and for proposals to immunize women with human chorionic gonadotropin for fertility control.

Interactions of sex steroid hormones with proteins with receptor properties in the cell sap from sex steroid-responsive tissues have been studied this year. A protein with properties of a receptor for testosterone has been demonstrated in cell sap from glomerulosa cells derived from preantral follicles of estrogen-primed hypophysectomized immature female rats. In addition, it has been shown that testosterone is translocated to the nuclei of these cells where the steroid is bound to a macromolecule. It is thought that these observations are consistent with a role of androgens in regulating follicular growth and atresia in mammalian ovaries. The cell sap from adrenal cortical cells of the guinea pig have been shown to contain a receptor protein for estrogen, providing a basis for the participation of this sex steroid hormone in the control of adrenal cortical androgen synthesis, increases which are concommitant with pubescence in guinea pig and humans. Two important drugs which cause gynecomastia as a side-effect were shown to interact with cytosol receptors in breast tissue. The first, spironolactone, inhibits the binding of testosterone to its receptor in this cytosol, whereas the second, digitalis, acts as a weak estrogen in the same system. The current hypothesis being studied in light of these observations is that alterations in testosterone/estrogen ratios in the target tissue are the pathophysiologic basis for gynecomastia. Methods used in these studies of hormone-receptor interactions in vitro will be applied to studies of disorders of the phenotypic expression of sexual development in such diseases as gynecomastia and hirsutism.

Interactions of labeled gonadotropin with receptors in particulate and soluble fractions isolated from homogenates of testicular Leydig cells have been further studied. Particulate gonadal fractions have been used in affinity chromatography systems to enhance the reactivity of labeled FSH and hCG for use in radioligand-receptor assays, and to develop methods sensitive enough to apply to biologic fluids. Radioligand-receptor assays employing gonadal particulate fractions have been used to compare the biological activities of labeled and unlabeled hormone, making it possible to calculate the specific activity of labeled peptide hormone. This development will contribute to increased accuracy in calculating numbers and affinities of receptor sites during changes such as growth and development. Moreover, careful analysis of the rate constants of association, dissociation and degradation of hormone and receptors in these in vitro systems increases the accuracy of calculation of receptor site turnover rates. Computer-based programs have been developed for making these calculations with appropriate corrections for the specific activity, biological activity, binding properties and degradation of the labeled hormone.

The solubilization and purification of gonadotropin receptors of testis and ovary have been extended to isolation of LH/hCG receptors by affinity chromatography, in a state approaching theoretical purity. Application of this approach to large-scale purification of the receptor sites is being performed in the hope of isolating sufficient receptors for structural, immunological and biological studies. Similar procedures have been applied to solubilization of FSH receptors from the testis tubules. The relationships between solubilized LH receptors and adenylate cyclase were studied in detergent extracts of testis and ovary, and suggest that a physical association between receptors and cyclase may exist in solution. The availability of purified LH receptors, and the analysis of receptor-cyclase interactions, should increase knowledge of the basic mechanisms of gonadotropin action and receptor activation in gonadal target cells.

Studies of interactions of gonadotropins with suspensions of intact single cells have also continued. Suspensions of intact Leydig cells from rat testes have been shown to respond to physiologic concentrations of LH and hCG by secretion of testosterone into the incubation medium. This action of gonadotropins on steroid hormone secretion has been found to require continuous RNA and peptide synthesis by these cells. In suspensions of these cells responding to hCG, the cyclic AMP dependent protein kinase was resolved into two forms corresponding to Types I and II holoenzymes of myocardial and adipose tissue. The role of these two forms of protein kinase in the mechanism of action of gonadotropins in stimulating steroid hormone synthesis can now be examined.

Ovarian cell suspensions were prepared from corpora lutea excised surgically during varying times in the menstrual cycle, and during gestation in pregnant rhesus monkeys. Progesterone secretion in response to stimulation with hCG was shown to decline with age of the corpus luteum during the cycle and during gestation. Thus, hormonal responsiveness in vitro corresponded to declining levels of progesterone during the menstrual cycle, and to decreasing importance of the corpus lutuem as a source of progesterone during gestation in the rhesus monkey. Addition of estrogen to the incubation medium was shown to inhibit response of the luteal cells to hCG, suggesting that estrogen modulates the response of the corpus luteum to gonadotropins. This cell preparation provides an excellent model system for the study of substances with luteolytic potential for fertility control in mammals.

Many of the methods and many of the reagents used for studies of model systems have been applied to studies of the physiology of the reproductive process in the whole animal. Beginning with fertilization of the egg, pronuclei were shown to be essential for formation of annulate lammelae in parthogenetically activated rabbit eggs, indicating that sperm are not required for this process. Cleavage planes were studied in rabbit, mouse and rat eggs fertilized in vitro. These studies show that in eutheria, as in vertebrates, the second cleavage plane is perpendicular to the first, resolving an apparent paradoxical discrepancy in these two orders.

Fetal, maternal and placental interactions have been studied during pregnancy in rhesus monkeys in which maternal and fetal hypophysectomy and fetectomy were performed in different sequences and combinations. It was shown that the placenta secretes an adrenocorticotrophic substance which maintains adrenal weight and prevents decline in maternal serum cortisol levels following maternal hypophysectomy. Furthermore, the maternal hypophysis appears to contribute to the control of serum estradiol levels in both fetal and maternal compartments during gestation in this species. The morphological and functional consequences of these surgical ablative procedures on other pituitary-dependent target organs are being examined.

Reagents developed for detecting and measuring chorionic gonadotropin in rhesus monkey serum have been applied to detection of the hormone in these fluids from other primates. To date, these reagents have been found useful in diagnosing pregnancy in baboons, marmosets and chimpanzees, in addition to their use in reproduction research in the rhesus. More than 400 kits containing reagents for 40 tests each have been distributed to reproductive biologists around the world. False-positive and false-negative tests are less than five percent in extensive field trials reported to date.

Radioimmunoassays specific for rhesus monkey follicle stimulating hormone have been developed, and an assay for LH in rhesus plasma has been adapted for use in our laboratories. These reagents have been used to measure hormonal concentrations in serum specimens collected from monkeys in which the dominant follicle was destroyed by cautery on the 8th to 12th day of the cycle, or the corpus luteum excised on days 15-17. Results indicate that by the 8th day of the cycle, the follicle destined to ovulate has been selected. Furthermore, the time and site of new follicle growth are not random events but appear to be locally determined by either the dominant follicle or corpus luteum in cycling rhesus monkeys.

Studies have been initiated to examine mechanisms by which nutritional factors influence ovarian function in mammals. For this purpose, gonadal and gonaductal morphology and sex steroid concentrations have been examined in hypophysectomized immature female rats given mixtures of FSH and hCG during treatment with metabolic antagonists for certain of the B complex vitamins. Preliminary results indicate that sex steroid synthesis is inhibited in animals receiving such antimetabolites.

Mechanisms regulating biosynthesis and turnover in gonadal receptors have been studied in testes of normal and hypophysectomized adult rats. While LH receptors declined rapidly to levels approximately 40% of those in control animals, FSH receptors did not decline until three weeks postoperatively. Furthermore, the loss of LH receptors was not uniformily distributed among all of the Leydig cells in the testes of hypophysectomized rats. Most interestingly and most importantly, the loss of receptors was accompanied by a decline in steroidogenic response to luteinizing hormone without any decrease in the cyclic AMP response, which was in fact increased. Finally, no incorporation of labeled aminoacids into ovarian LH receptors could be shown in animals pulsed during the rapid phase of gonadotropin-induced increase in LH receptor activity, suggesting a discrepancy between biosynthesis and activity of the receptors. Results of these fundamental studies are potentially very important for understanding control of both sex steroid hormone production and the gametogenesis with which it is associated in the gonads.

The synthesis and secretion of testosterone by suspensions of intact Leydig cells responding to hCG have been utilized to develop very sensitive in vitro bioassays for LH/hCG in specimens of human biological fluids. In specimens collected from normal women throughout the menstrual cycle, the ratio of biologic to immunologic potency is unity, whereas in specimens collected from men and postmenopausal women, the ratios are 2 or 3 to 1. The structural basis for these discrepancies in potency will be investigated. This in vitro bioassay system has tremendous potential for studying the structure-function relationships in chemically modified preparations of luteinizing hormone.

Radioimmunoassays have been used to study interactions of the central nervous system, pituitary, and gonads in man and other primates. It has been shown that estradiol is a dominant regulator of pituitary LH secretion in the normal male. However, a quantitatively less impressive, but distinctive androgenic effect, mediated by testosterone, has also been demonstrated. The blood-CSF barrier has been shown to be a potential control point in the sex steroid hormone-brain-pituitary interactions. Preliminary studies in an animal model system are consistent with the presence of a nonsteroidal substance of testicular origin (inhibin) which regulates pituitary gonadotropin secretion, particularly that of FSH.

Other projects concerned with mechanisms of hormone action and secretory processes have been focused upon the functions of the adrenal gland. At the subcellular level of organization, chromaffin granules isolated from bovine and chicken adrenal medullae have been shown to contain adenylate cyclase, which is activated only during release of the stored hormone contents of these granules. Furthermore, cyclase activation appears to be associated with alterations in transmembrane potential which is a concomitant of hormonal release. In addition to the contributions to our understanding of the molecular basis for control of hormonal secretion, these studies provide a rational basis for designing drugs for treating a variety of diseases in which apparent hormonal deficiencies result from failure to secrete rather than failure to synthesize these substances.

In the adrenal cortex, extensive studies on angiotensin II receptors and the control of aldosterone synthesis in zona glomerulosa cells have continued. The properties of angiotensin II receptors in dog and rat adrenal homogenates and cell suspensions were defined, and occupancy of receptors by angiotensin II was shown to be correlated with glomerulosa cell activation and aldosterone production in vitro. The affinity and number of adrenal receptors were shown to be influenced by dietary electrolyte intake, and conditions were established for in vitro and in vivo studies on adrenal sensitivity to angiotensin II and other stimuli during altered sodium balance. In dispersed adrenal cells from dog and rat adrenals, important interactions between potassium and angiotensin II were observed at physiological levels of these regulators of aldosterone secretion. Extracellular sodium concentration was also shown to exert a significant direct action upon aldosterone production in vitro. The sensitivity of isolated adrenal cells to angiotensin II and ACTH has been extended to the picogram level, offering the potential for development of bioassays for glomerulotropic factors as well as for studies on the actions of such stimuli at physiological concentrations. The actions of angiotensin II agonist and antagonist derivatives were also analyzed in terms of receptor binding in inhibition of aldosterone responses. This combination of methods has been particularly useful for evaluation of angiotensin II antagonists, and has shown that the derivatives formed by aliphatic substitution of the C-terminal phenylalanine residue of angiotensin II are potent antagonists at the adrenal level. These methods have also been of value in demonstrating the agonist activity of angiotensin antagonists, and revealed that increasing the length of the C-terminal aliphatic side-chain caused increased agonist potency as well as increased binding affinity for the angiotensin receptor sites. In addition to their relevance to the mechanisms of peptide hormone action and the physiological control of aldosterone secretion, these studies provide basic information about hormonal and ionic interactions which become disordered in abnormal conditions of aldosterone secretion, as in certain forms of hypertension and renal disease. In particular, the application of adrenal cells to in vitro bioassay of circulating trophic factors has considerable potential for studies on human physiological responses and abnormal states of aldosterone secretion.

In the clinical area, continuing studies of the syndrome of anorexia nervosa in young women have led to the conclusion that the disease has a large component of disorered hypothalamic function. The drug, cyproheptadine, an anti-serotonergic agent, has been used in a double-blind treatment protocol in an attempt to stimulate appetite in these young women. This study is nearing completion and results indicate that such therapy has potential for improving the results of treatment of this disorder.

In summary, studies that range from the molecular level to the intact organism have been undertaken by scientists in the Reproduction Research Branch during the past year. The results constitute an impressive contribution to knowledge of reproductive processes, with tremendous potential for applications to diagnosis and treatment of human disease, and for use in the search for alternative methods for reversible voluntary fertility control.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00022 03-RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Renin-Angiotensin System and Aldosterone Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

1) Research Foundation, Cleveland Clinic, Cleveland, Ohio
2) Hypertension Clinic, D. C. General Hospital, Department of Medicine Georgetown University

LAB/BRANCH
Reproduction Research Branch

SECTION
Section on Hormonal Regulation

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.8	3.8	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

This program is composed of an inter-related group of studies on the role of the renin-angiotensin system in hypertension and in the regulation of aldosterone secretion by the zona glomerulosa of the adrenal gland. Individual areas of research include the characterization and regulation of angiotensin II receptors, the actions of angiotensin II and other regulators of aldosterone secretion, the production and metabolism of angiotensin II, and the relationship of renin, angiotensin II and aldosterone to certain forms of clinical hypertension.

Objectives.

The objectives of this project are to clarify the role of the renin-angiotensin system in the regulation of aldosterone secretion in physiological and pathological conditions; to characterize the angiotensin II receptors and their role in the activation of adrenal steroidogenesis; to elucidate the mechanisms of action of angiotensin II and other regulators of aldosterone secretion in the zona glomerulosa; and to evaluate the significance of the renin-angiotensin system and aldosterone in certain forms of clinical hypertension.

Methods Employed.

Radioimmunoassays of renin activity, angiotensin II, aldosterone, corticosterone, cortisol, and cyclic nucleotides. Binding studies with ^{125}I- and ^{3}H-angiotensin II in adrenal cells and membranes. In vivo and in vitro stimulation of aldosterone responses by adrenal cells. Measurement of agonist and antagonist activities of angiotensin II analogues.

Major Findings.

1. Angiotensin II Receptors of the Adrenal Gland. The binding sites for angiotensin II previously identified in bovine and rat adrenal gland have been more extensively studied in the dog and rat adrenal cortex. In each species examined, the biological specificity of the receptors has been demonstrated by their affinity for angiotensin II agonist and antagonist analogues. The kinetic and equilibrium constants for angiotensin II binding were determined in adrenal cell membranes and in isolated glomerulosa cells. The kinetics of binding were rapid and temperature-dependent, and the binding affinity of angiotensin II receptors in isolated glomerulosa cells was high ($Ka = 10^{10}\ M^{-1}$) and commensurate with physiological levels of angiotensin II in the circulation ($2\text{-}5 \times 10^{-11}$ M).

The actions of dietary electrolyte intake on adrenal receptors and the renin-angiotensin system were further analyzed in rats maintained on modified sodium and potassium diets. Angiotensin II receptors were increased in adrenal glands of rats on low sodium or high potassium diets, and were decreased by high sodium or low potassium intake. The rise in receptors during sodium deficiency was accompanied by an increase in the number of sites per glomerulosa cell. When sodium intake was varied, the adrenal content of angiotensin II receptors was directly proportional to the circulating levels of renin and angiotensin II. Conversely, variations in potassium intake were characterized by an inverse relationship between renin-angiotensin II levels and adrenal receptor sites.

Adrenal receptor sites for angiotensin II were reduced in rats sacrificed under ether or pentobarbital anesthesia, which markedly increased plasma renin activity and caused occupancy of receptors by endogenous angiotensin II. Acute elevation of blood angiotensin II by infusion of the synthetic octapeptide likewise decreased the adrenal content of free angiotensin II receptors. However, the number of adrenal angiotensin II receptors was also decreased by nephrectomy in sodium-deficient animals, suggesting that circulating angiotensin II acts to maintain the receptor sites of the adrenal cortex.

2. Radioligand-Receptor Assay of Angiotensin II and Analogues. The binding of ^{125}I-angiotensin II to particulate fractions of bovine and dog adrenal cortex was applied to the development of radioligand-receptor assays for angiotensin II and related peptides. The biological relevance of angiotensin II binding to such adrenal preparations was illustrated by the close correlation between the binding-inhibition potencies and biological activities of angiotensin fragments and agonist analogues. An important practical application of such radioligand assays was illustrated by the evaluation of a series of angiotensin II antagonists. These peptides showed binding activities which were closely correlated with their biological activities as antagonists of angiotensin II actions upon smooth muscle contraction and aldosterone production by the adrenal gland. Thus, the adrenal binding system provides a simple and rapid screening method for evaluation of the binding activity of angiotensin II analogues with potential antagonist activity. Used in conjunction with the adrenal cell system for assay of the biological effects of angiotensin analogues on aldosterone production, the radioligand assay gives a quantitative measure of the role of receptor binding affinity in the biological action of angiotensin agonists and antagonists. The radioligand assay has also been of value in the measurement of specific activity of radioiodinated and tritiated angiotensin II tracers, in terms of the biological activity of the unlabeled peptide. Such estimates are essential for the derivation of accurate binding constants from data generated during binding studies with labeled angiotensin II.

3. Development of Isolated Adrenal Glomerulosa Cell Systems with High Sensitivity to Angiotensin II and ACTH. To provide an in vitro system for analysis of the mechanisms of action of angiotensin II and other factors upon aldosterone production, a method for preparation of isolated zona glomerulosa cells was developed and optimized. By controlled dispersion of dog or rat adrenal glomerulosa with collagenase, it is possible to prepare viable adrenal cells which produce aldosterone in response to angiotensin II and other regulators in vitro. The dog and rat glomerulosa cells respond to angiotensin II in concentrations as low as 10^{-11} M, and show a maximum steroid response of 3×10^{-10} M angiotensin II. This is the first development of an adrenal cell system which responds to angiotensin II concentrations in the circulating range. Such cells are ideal for studies on the mechanism of action of angiotensin II at physiological concentrations, and also form a potential assay for application to adrenal disorders such as primary aldosteronism caused by bilateral adrenal hyperplasia. The rat adrenal cells were found to be more responsive to ACTH than to angiotensin II, in keeping with the marked effects of stress on aldosterone levels in this species, and produce aldosterone in response to ACTH concentrations as low as 10^{-12} M.

The adrenal cells gave identical aldosterone responses to angiotensin II and the [Des-Asp^1]-heptapeptide, whereas the C-terminal hexapeptide and N-terminal heptapeptide fragments were almost completely inactive, in keeping with their lack of activity upon adrenal and muscle in vivo. By contrast, the [Sar^1] derivative of angiotensin II was almost 10 times more active than angiotensin II in stimulating aldosterone production, in keeping with its enhanced potency upon smooth muscle contraction.

4. Actions of Angiotensin II Antagonists on Aldosterone Production In Vitro. The biological activities of angiotensin II antagonist analogues upon basal and angiotensin II-stimulated aldosterone production were analyzed in the isolated adrenal cell preparation. The [Sar^1, Ile^8] derivative was the most potent antagonist peptide, but also displayed significant agonist activity at concentrations required to inhibit the steroidogenic effect of angiotensin II. The [Sar^1, Ala^8] and [Sar^1, Gly^8] derivatives were less potent than the [Ile^8] compound on a molar basis, but did not exhibit agonist activity and were therefore more effective antagonists of angiotensin II. These results indicate that increased length of the aliphatic side chain of C-terminal substituted antagonists is accompanied by increased affinity for the receptor sites, but also by increased agonist activity upon aldosterone synthesis.

These studies also demonstrated that the antagonist activities of angiotensin II analogues upon adrenal cells was correlated with their antagonist activities upon smooth muscle. Also, the heptapeptide antagonists were less effective than the octapeptide antagonists in blocking the actions of angiotensin II upon adrenal cells. Thus, the angiotensin II receptors in muscle and adrenal show generally similar responses to angiotensin II agonists. Also, the postulated role of the angiotensin II heptapeptide as an intermediate in the action of angiotensin on adrenal steroidogenesis has not been supported by these studies and others performed in the isolated adrenal cell preparation. The production of aldosterone by isolated glomerulosa cells provides a highly sensitive and biologically relevant response for measurement of the agonist and antagonist properties of angiotensin II analogues upon the adrenal gland.

5. Angiotensin II-Receptor Interactions and Steroidogenesis in Isolated Adrenal Cells. Dispersed adrenal cells were also of value for studies on angiotensin II binding to cellular receptors and comparison of binding with stimulation of steroidogenesis. By such methods, occupancy of angiotensin II binding sites could be correlated with subsequent increases in aldosterone production, indicating the biological function of these sites as receptors for angiotensin II. Also, the rate constants for binding of angiotensin II agonists and antagonists to intact cells were concordant with their binding affinities in cells and homogenates, and the [Sar^1] derivative of angiotensin II was shown to have a seven-fold higher ka than angiotensin II. These methods provide an approach to the analysis of differential kinetic properties in the interactions between angiotensin II agonists and antagonists at the target cell level.

6. Mechanisms of Action of Angiotensin II and ACTH Upon Aldosterone Production. The biochemical pathway by which angiotensin II stimulates aldosterone production in the zona glomerulosa has never been elucidated. It is expected that the dispersed adrenal cell preparation will be of value for this purpose. The zona glomerulosa responds to ACTH and increased potassium concentration, as well as to angiotensin II, with increased aldosterone production. The action of ACTH is probably via stimulation of adenylate cyclase and cyclic AMP formation, since the response can be elicited by addition of dibutyryl cyclic AMP, and increased production of cyclic AMP during ACTH stimulation has been demonstrated in the isolated canine glomerulosa cells. However, no change in cyclic AMP or cyclic GMP levels was measured in the

same cells during stimulation of aldosterone production by angiotensin II. Thus, the mechanism of action of angiotensin II upon the adrenal glomerulosa does not involve a change in cyclic nucleotide concentrations within the target cell. A change in ion transport as a possible alternative mechanism is presently under investigation, and preliminary studies on [Na^+, K^+] dependent ATP'ase in the adrenal have shown no change during angiotensin II action.

7. Interaction of Peptide Hormones and Cations Upon Aldosterone Regulation. The sensitivity of the adrenal cell system to stimuli of aldosterone has allowed the demonstration of previously unrecognized interactions between peptide regulators and potassium and sodium. In particular, changes in the extracellular potassium level within the physiological range have been shown to significantly modulate the sensitivity and magnitude of steroid response to angiotensin II. Also, changes in extracellular sodium concentration were found to influence the action of potassium upon aldosterone production, and to have a significant direct action upon the steroidogenic activity of the isolated glomerulosa cells. Such interactions between peptide and ions at physiological levels constitute important and subtle regulatory mechanisms which could operate in vivo to determine the final adrenal response to simultaneous changes in renin-angiotensin activity and electrolyte balance.

8. Low-Renin Hypertension. A longitudinal study on low-renin hypertension, performed in collaboration with the Hypertension Clinic, D. C. General Hospital, has been continued. Additional patients have been classified on the basis of plasma renin and urinary sodium excretion, and added to the original groups studied to determine whether the incidence of heart attacks and strokes was related to the renin status in hypertension. Also, the earlier retrospective study, which demonstrated that low renin hypertensives were not protected against vascular complications, is being followed by a prospective analysis of our patient groups. The consistency of plasma renin studies with prolonged treatment, and the value of plasma aldosterone in biochemical profiling of hypertensive patients, are also being examined.

Significance.

This project is providing new information about the control of aldosterone secretion and the role of the renin-angiotensin system in adrenal regulation and in certain forms of hypertension.

Proposed Course.

The metabolism of angiotensin II by adrenal cells will be studied in vitro, to examine the fate of the peptide at the target cell and the role of peptide fragments in aldosterone stimulation. The role of ion shifts in angiotensin action, and the steroid biosynthetic changes induced by stimuli of aldosterone secretion will also be analyzed. In vivo binding studies with labeled angiotensin II and analogues will be performed, and the early effects of sodium restriction and excess upon angiotensin II binding and adrenal sensitivity will be examined in vitro.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00025-02 RR

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

α-1-Fetoprotein as an Indicator of Fetal Well-being

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. W. Tullner	Senior Investigator	RRB	NICHD
OTHER:	G. D. Hodgen	Chief, Section on Endocrinology	RRB	NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Reproduction Research Branch

SECTION

Section on Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

AFP was first detectable in maternal serum of pregnant monkeys (Macaca mulatta) at 6-8 weeks gestation (gestational length 24 weeks) using a sensitive (10 ng/ml) and specific radioimmunoassay. AFP concentrations rose rapidly and were maintained at 100-200 ng/ml for about 10 weeks. During the 3 weeks preceding parturition, serum AFP levels declined. In babies delivered naturally or by hysterotomy at term, serum AFP levels ranged from 19,000 to 27,000 ng/ml in contrast to maternal levels of <10 to 60 ng/ml. Maternal hypophysectomy produced a marked rise in maternal serum AFP at 6-7 days after surgery in 3 of 4 animals. Fetectomy at 10 weeks was followed by a similar rise and AFP was present in maternal serum at 5-6 weeks after removal of the fetus. Since the half-life of serum AFP was found to be 2½-3 days, these results indicate retention or synthesis of AFP in an extra-fetal site as well as an effect of maternal stress on AFP levels.

Project Description:

Our investigations on normal and experimental rhesus monkeys show that maternal serum AFP levels maintain a reproducible pattern during pregnancy with earliest detection at 6-8 weeks (using a sensitive radioimmunoassay with antisera specific for rhesus monkey AFP). Thereafter, AFP concentrations increase rapidly and are maintained in a range of 100-200 ng/ml for about 10 weeks with a decline during the 3 weeks prior to parturition. With the approach of parturition, fetal serum AFP can be markedly elevated despite baseline levels in maternal circulation. The independent control of AFP levels in maternal and fetal serum compartments is supported further by our finding that AFP levels were sustained for 2 weeks following removal of the fetus even though experimental data show a half-life of 2½-4 days for AFP. This confirms previous findings in monkeys with liver cancer. We found a marked rise in maternal serum AFP after fetectomy and maternal or fetal hypophysectomy, with AFP present in maternal serum several weeks after removal of the fetus. Thus, elevated serum AFP concentrations were associated with trauma or stress in the absence of the fetus. Amniotic fluid levels in two monkeys carrying dead fetuses had AFP concentrations several hundred-fold higher than comparable controls. Repeated confirmation of the amniotic fluid results may provide a useful method for the diagnosis of fetal distress or death.

We plan to continue evaluation of differences in AFP concentrations in amniotic fluid of normal monkeys and those involved with fetal distress. Further, the role of surgical trauma in producing an increase in maternal serum AFP levels will be examined.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00026-02 RR

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Parthenogenetic Activation of Mammalian Oocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. J. Gulyas	Senior Investigator	RR NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Reproduction Research Branch

SECTION
Section on Endocrinology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Parthenogenetically activated rabbit eggs were used to test whether or not formation of annulate lamellae (AL), a cytoplasmic organelle produced after the formation of pronuclei in fertilized eggs, is dependent on the presence of an intact nucleus. It was shown that AL formation occurs in parthenogenetic rabbit eggs as long as a nucleus is formed after artificial activation.

PHS-6040

The objective of this project was to determine whether or not parthenogenetically activated rabbit eggs are capable of forming annulate lamellae (AL) in the absence of the fertilizing sperm and whether or not the presence of a nucleus is essential in this process. Unfertilized rabbit eggs were cold activated by storing them at 10°C for 24 hrs. Following cold-shock the eggs were cultured for 24 h and then prepared for electron microscopic observations. Two-thirds of the cold-activated eggs formed one or two pronuclei. In addition, some eggs formed several subnuclei. The remainder third of the eggs failed to form any type of nucleus. AL were present only in those parthenogenetic eggs which formed pronuclei or subnuclei. AL were absent in the control and the non-activated eggs, each of which failed to form nucleus. These findings establish that the sperm is not required for AL formation, however, this process requires the presence of an intact nucleus.

This project on parthenogenetic activation of mammalian oocytes will be continued during the coming year. Two separate procedures have been tested in this laboratory for effectiveness in obtaining parthenogenones. Cold-shock treatment of rabbit eggs was effective to induce nucleus formation, but the cortical granules were not released. Because abnormal cortical reaction in the parthenogenones could have adverse effects on further development of the eggs, we tried a second approach, electrical stimulation, on rabbit, hamster and mouse eggs. The results of this study showed that although electrical stimulation can evoke some favorable responses in the mouse and the hamster, it is not an ideal parthenogenetic agent for either of the species tested because some of the reactions of the eggs can be detrimental to further development. Our preliminary results indicate that ionophores can be utilized effectively to release the cortical granules. These agents might cause fewer side effects. A single parthenogenetic agent may not be able to elicit the whole chain of reactions, and for this reason we will consider utilizing combination of several agents.

Publications:

Gulyas, B.J.: The dependence of annulate lamellae formation on the nucleus in parthenogenetic rabbit eggs. Cell Tiss. Res., 162: 475-481, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00028-02 RR

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Influence of Nutritional Factors on Pituitary and Gonadal Hormones in Rats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. E. Nixon	Senior Investigator	RR	NICHD
OTHER:	G. T. Ross	Chief, Reproduction Research Branch	RR	NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Reproduction Research Branch

SECTION

Section on Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.25	.25

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to study pituitary and gonadal function as reflected in the secretion and/or organ content of specific hormones and the affect of nutritional deficiencies on these functions. The topics of current interest are: 1) The secretion of ovarian steroids by normal and pyridoxine-deficient immature hypophysectomized rats administered an hCG-oFSH regimen; 2) The effect of these regimen on the microscopic and ultrastructure of the ovary; 3) The improvement of the response of the animal preparation; 4) The establishment (in-house) of biochemical tests of nutritional deficiencies.

Project Description:

Objectives

The objectives of this project are to study pituitary gonadotropic and gonadal steroid hormone synthesis and secretion as affected by vitamin and protein-calorie deprivation.

Methods

Hypophysectomized and castrate animals which have received tropic hormones are used to test the affect of nutritional deficiencies on target organ function.

Specific radioimmunoassay of hormonal products serves as the observed end point of functional activity.

Major Findings

The ovarian and uterine growth and serum estrone, estradiol and progesterone response to an hCG-oFSH regimen in hypophysectomized immature rats is dose related to the administered FSH. A pyridoxine deficiency induced with deoxypyridoxine (50 μg/day or more) tends to decrease ovarian growth and serum estradiol and progesterone responses.

Significance to Biomedical Research

The nutritional inadequacy of the diet of many citizens is a matter of record. In addition, the widespread use of steroid containing contraceptive pills with the reported increased need of specific dietary constituents, especially some vitamins, makes it desirable to understand the relationships between the reproduction-associated endocrine system and nutrition.

Proposed Course

It is proposed that work will continue to develop an animal model which gives a consistent response to tropic hormones.

The effects on model response will be determined for other vitamins including riboflavin and nictinamide, as well as pyridoxine, and for protein also.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00029-02 RR

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Ultrastructure of Primate Corpus Luteum

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. J. Gulyas	Senior Investigator	RR	NICHD
OTHER:	G. D. Hodgen	Chief, Section on Endocrinology	RR	NICHD
	W. W. Tullner	Senior Investigator	RR	NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Reproduction Research Branch

SECTION
Section on Endocrinology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The fine structural aspect of primate (Macaca mulatta) corpus luteum (CL) was examined at termination of pregnancy. Corpus luteum was obtained from 1) intact, 2) hypophysectomized and 3) fetectomized monkeys. The fine structural observations showed that the CL from hypophysectomized and fetectomized animals showed only minor variation from the intact ones, and that the granulosa lutein cells in all three groups of animals possess fine structural characteristics essential for steroidogenesis.

In the monkey (Macaca mulatta) the corpus luteum (CL) undergoes a transient regression during midpregnancy, which is followed by structural and functional rejuvenation near term. This investigation was designed to determine whether morphological rejuvenation of the CL occurs despite elimination of maternal pituitary by hypophysectomy, or products of fetal metabolism by fetectomy. Completeness of hypophysectomy was confirmed from histological examination of serial sections of the sella turcica. CL was prepared for fine structural examination by routine electron microscopic techniques. These observations show that granulosa lutein cells from all groups had fine structural characteristics indicative of active steroidogenesis. Cells of the corpus luteum from hypophysectomized animals had smaller mitochondria, often associated with lamellar structures. Mitochondria in luteal cells from fetectomized monkeys had large osmiophilic inclusions. In these cells agranular endoplasmic reticulum and Golgi were more numerous as compared to luteal cells from intact animals. Onset of luteolysis was most noticeable in the intact and hypophysectomized animals, but not so in the fetectomized ones. We concluded that the fine structure of the CL from the hypophysectomized and fetectomized monkeys showed only minor variation from the intact ones. These findings support the hypothesis of others that the source of hormonal stimulation for the rejuvenation of the CL in this primate may be the placenta.

This project is being continued in collaboration with others in this section. Our further objectives are to determine if these cells are responsive to gonadotropin stimulation in an in vitro system.

Publications:

Gulyas, B.J., Yuan, L., Tullner, W.W. and Hodgen, G.D.: The fine structure of corpus luteum from intact, hypophysectomized, and fetectomized pregnant monkeys (Macaca mulatta) at term. Biol. Reprod., (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00030-02 RR

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Characterization of Patterns of Chorionic Gonadotropin Production in Primates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. D. Hodgen	Chief, Section on Endocrinology	RR	NICHD
OTHER:	H. C. Chen	Senior Investigator	RR	NICHD
	C. K. Turner	Biol. Lab. Tech.	RR	NICHD
	D. L. Barber	Biol. Lab. Tech.	RR	NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Reproduction Research Branch

SECTION

Section on Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

As described in previous reports, a hemagglutination inhibition test suitable for the diagnosis of pregnancy in macaques has been developed in this laboratory. The Subhuman Primate Pregnancy Test kit, distributed under the aegis of the Contraceptive Development Branch, Center for Population Research, NICHD, has been sent to primate facilities all over the world. To date, more than 500 kits have been delivered, sufficient for 25,000 pregnancy tests. In order to realize the full potential of this kit, we have undertaken a series of studies to determine the interval for most reliable detection of urinary chorionic gonadotropin in baboons, marmosets, chimpanzees and squirrel monkeys. The first 3 of these are now complete.

Project Description:

The need for early, reliable diagnosis of pregnancy in primates derives from their importance as surrogates for man in biomedical research where the earliest possible diagnosis of pregnancy is required, such as investigations of teratogenic agents and improving the efficiency in breeding management situations.

Urine specimens were collected from baboons, marmosets and chimpanzees at various intervals throughout pregnancy. Using the Subhuman Primate Pregnancy Test kit, these specimens were tested in this hemagglutination inhibition test to determine the interval during which investigators could expect a reliable diagnosis of pregnancy.

The results indicate that this kit provides an accurate diagnosis of pregnancy by 17 days after fertilization in the baboon and the test remains reliable until day 30. For the marmoset, the test is most useful in the interval from 25 days after fertilization until day 70. Pregnant chimpanzees excrete chorionic gonadotropin at detectable levels from day 20 until near 133 days in gestation. As these results illustrate,the Subhuman Primate Pregnancy Test kit is a valuable laboratory tool for use with a variety of species. However, its proper application requires careful testing of each type of primate because of widely different patterns of chorionic gonadotropin excretion.

By comparison with traditional procedures for diagnosis of pregnancy in primates, including palpation and bioassay, this hemagglutination inhibition test is obviously the method of choice. This test yields definitive results within 2 hours, requires only 2 drops of neat urine, and can be conducted adjacent to the animal quarters without elaborate instrumentation.

Future studies will extend application of the Subhuman Primate Pregnancy Test kit to other species, such as squirrel monkeys, since these "new world" primates are more readily available, at lower cost to biomedical research, than rhesus monkeys and apes.

Publications:

Hodgen, G. D.: Comparative Primate Reproductive Endocrinology: Advancements Important in Domestic Breeding Programs. Lab. Anim. Sci. 25: 793-797, 1975.

Hodgen, G. D. and Niemann, W. H.: Application of the Subhuman Primate Pregnancy Test Kit to Pregnancy Diagnosis in Baboons. Lab. Anim. Sci. 25: 757-759, 1975.

Hodgen, G. D., Wolfe, L. G., Ogden, J.D., Adams, M. R., Descalzi, C.C. and Hildebrand, D. F.: Diagnosis of Pregnancy in Marmosets: Hemagglutination Inhibition Test and Radioimmunoassay for Urinary Chorionic Gonadotropin. Lab. Anim. Sci. In Press.

Publications: (Continued)

Hodgen, G. D., Niemann, W. H., Turner, C. K., and Chen, H.C.: Diagnosis of Pregnancy in Chimpanzees using the Subhuman Primate Pregnancy Test Kit. J. Med. Primatol. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00031-02 RR

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Hormonal Interrelationships between Fetus, Mother and Placenta in Primates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. D. Hodgen	Chief, Section on Endocrinology	RRB	NICHD
OTHER:	W. W. Tullner	Senior Investigator	RRB	NICHD
	B. J. Gulyas	Senior Investigator	RRB	NICHD
	W. E. Nixon	Senior Investigator	RRB	NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Reproduction Research Branch

SECTION

Section on Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Peripheral serum cortisol levels were measured throughout gestation in 5 intact pregnant rhesus monkeys (Macaca mulatta) and 3 hypophysectomized-fetectomized monkeys, leaving the placentas in situ and viable. Hypophysectomy at 70 to 73 days after fertilization caused a marked decline ($p<0.01$) in serum cortisol concentrations to about 1/2 the preoperative level. These monkeys were fetectomized at 107 to 114 days without further reduction in circulating cortisol levels. In hypophysectomized-fetectomized monkeys, either surgical removal of the placentas near term or abortion was followed by a rapid decrease in peripheral cortisol to undetectable concentrations. Their cortisol levels were 5 to 12 times higher in left adrenal venous effluent than in peripheral circulation on the day of placental delivery. The presence of a viable placenta protected against the extensive adrenocortical involution seen in nonpregnant hypophysectomized monkeys ($p<0.01$). Fetectomy, alone or in combination with hypophysectomy, did not alter left adrenal gland weights from those of intact pregnant monkeys. Thus, continued cortisol secretion and maintenance of adrenal weight in hypophysectomized-fetectomized monkeys, in the presence of a functional placenta, supports the existence of a placental adrenocorticotropin in this primate.

Project Description:

Evidence both in primates and rodents has established that maternal adrenal gland weights are partially maintained when maternal hypophysectomy is performed during pregnancy. In contrast, removal of the hypophysis from nonpregnant females is followed by rapid involution of adrenal cortices. These findings led to speculation that the conceptus may produce an adrenocorticotropic agent capable of stimulating the maternal adrenal cortex during gestation. The passage of adrenocorticotropic hormone (ACTH) from the fetal pituitary across the placenta or secretion of an adrenocorticotropic substance by the placenta has remained controversial.

We have combined hypophysectomy and fetectomy in pregnant rhesus monkeys to eliminate both the maternal hypophysis and fetus from the model, leaving the placentas in situ and viable. Evidence is presented that the primate placenta may secrete an adrenocorticotropic substance which sustains, in part, cortisol secretion and maternal adrenal gland weight.

Maternal hypophysectomy, using parapharyngeal techniques, was performed in 3 rhesus monkeys (Macaca mulatta) at 70 to 73 days in gestation. Subsequently, the same monkeys were fetectomized at 107 to 114 days with the placentas maintained in situ. Five intact pregnant monkeys served as controls. These 8 monkeys were bled from the femoral vein at frequent intervals throughout gestation. On the day the placentas were delivered, a left adrenal venous specimen was collected.

Adrenal glands were removed for comparison of organ weights from 6 groups of animals either at the time when the placentas were delivered or as specified below. These groups consisted of: 1) intact, nonpregnant, 2) intact, pregnant, 3) pregnant, hypophysectomized, 4) pregnant, fetectomized, 5) pregnant, hypophysectomized and subsequently fetectomized, and 6) cycling monkeys which had been hypophysectomized for periods (92 to 99 days) corresponding to the hypophysectomized-fetectomized group.

Although serum cortisol concentrations in the intact, pregnant monkey varied markedly from one sampling interval to another and between individuals, there was a trend toward rising levels with advancing gestation. Excision of the maternal hypophysis caused a substantial decline in serum cortisol concentrations within 7 to 21 days. Subsequent fetectomy did not lead to a further decrement in the level of this hormone, which remained at about 1/2 the preoperative level. Surgical removal of placentas near term in 2 animals as well as abortion in another on the day after bilateral ovariectomy, was soon followed by a decline in circulating cortisol to undetectable levels. Adrenal vein blood from hypophysectomized-fetectomized monkeys contained 5 to 12 times more cortisol than peripheral levels on the day of placental delivery.

The protective influence of the placenta against adrenocortical insufficiency was also in evidence throughout the course of these experiments, in that no instances of coma were observed in the hypophysectomized monkeys carrying viable placentas. In contrast, nonpregnant females with their hypophyses removed were treated acutely with 100 mg of hydrocortisone sodium succinate iv during 11 separate comatose episodes to restore their motor faculties.

We have used a primate model with both the maternal pituitary and fetus eliminated to demonstrate continued stimulation of maternal adrenal cortisol secretion in the presence of a viable placenta. This finding provides experimental evidence for an adrenocorticotropin of placental origin.

In previous reports, we have examined the hormonal interrelationships within and between the maternal, fetal and placental endocrine compartments of the pregnant rhesus monkey (Macacca mulatta) by performing a variety of surgical ablations. These primate models have included comparisons of circulating levels of estrone, estradiol and progesterone after fetectomy or ovariectomy with those of intact pregnant monkeys. In addition, the role of the placenta in cortisol secretion by maternal adrenals of intact and hypophysectomized-fetectomized females has been studied.

As a continuation of these investigations, we have described the effects of maternal hypophysectomy followed by fetectomy on estrone, estradiol, and progesterone levels in peripheral blood throughout gestation, as well as the effects of maternal hypophysectomy alone on circulating levels of these hormones in both mother and term fetus or baby.

Three monkeys were hypophysectomized at 70 to 73 days gestation and later fetectomized at 107 to 114 days. Nine monkeys were hypophysectomized (anterior and posterior lobes) by a parapharyngeal approach (5) between 56-71 days gestation.

Although maternal plasma E_2 levels were temporarily reduced after hypophysectomy in the experiments reported here, this decline was followed by a rise within 4 to 5 weeks. However, the increased concentrations of E_2 were effectively terminated by fetectomy. Surgical removal of the fetus resulted in a progressive decline in plasma E_2 concentrations. Similarly, E_2 levels were basal in a monkey bearing a dead fetus. The decline in E_2 after hypophysectomy may be a consequence of surgical trauma with subsequent recovery. No marked alterations in plasma E_1 levels were seen after hypophysectomy. Plasma levels of progesterone either increased or remained at about the same level after ablation of the pituitary gland. Thus, the results indicate that surgical hypophysectomy per se exerts only temporary alterations in the normal patterns of plasma E_1, E_2 and P occurring during early pregnancy (70 to 110 days). There appeared to be no effect of hypophysectomy on the levels of E_1, E_2, or P in the maternal circulation of mothers which delivered babies at term. Low postpartum levels of these steroids in intact animals were associated with parturition. In hypophysectomized monkeys delivered by Cesarean section, blood levels of E_2 were elevated in comparison with those of intact mothers delivered surgically. In contrast, no such differences were found in the corresponding fetal or umbilical venous plasma. Among monkeys that delivered naturally, caval plasma of babies from intact mothers contained undetectable concentrations of E_2, whereas E_2 levels were significantly greater in infants from hypophysectomized females.

Thus, the major differences after hypophysectomy in this series of monkeys involved elevations in E_2, whereas E_2 levels were significantly greater in infants from hypophysectomized females.

The primary differences after hypophysectomy in this series of monkeys involved elevations in E_2 either (1) in the plasma of hypophysectomized mothers at the time of Cesarean delivery or (2) in babies of hypophysectomized mothers shortly after parturition. Except for these differences, the maternal hypophysis apparently is not a major factor in regulating the steroid hormonal milieu of pregnancy in this primate.

Extensions of these investigations using primate models will involve replacement therapy of high purified hormonal prepartions following surgical ablations.

Publications:

Hodgen, G. D., Gulyas, B. J., and Tullner, W. W.: Role of the Primate Placenta in Cortisol Secretion by the Maternal Adrenals. Steroids 26: 233-240, 1975.

Tullner, W. W., Gulyas, B. J., and Hodgen, G. D.: Maternal Estrogen and Progesterone Levels after Hypophysectomy in Early Pregnancy and in Term Fetuses or Newborn Monkeys. Steroids 26: 625-633, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00032-03 RR

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structure and Function of Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. S. Cohen,	Research Chemist	RRB	NICHD
Other:	H. Shindo	Visiting Fellow	RRB	NICHD
	W. Egan	Staff Fellow	RRB	NICHD

COOPERATING UNITS (if any)
E. Becker, Chief, LCP, NIAMDD, NIH.
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A. Lanir, Dept. of Biochemistry, Tel-Aviv University, Israel
E. Bucci, Dept. of Biochemistry, Maryland University Medical School, Baltimore

LAB/BRANCH
RRB

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 2.0	PROFESSIONAL:	OTHER:

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to elucidate the processes whereby proteins form unique native conformations and function selectively in solution. The method of choice is nuclear magnetic resonance (NMR) applied to directly observe resonances of individual atoms within the protein molecule. Most work to date has concentrated on the histidine C2 proton resonances as the only singly resolved proton resonances of a series of small stable proteins, e.g. ribonuclease, cytochrome c, myoglobin, using proton NMR. Recently we have been able to resolve for the first time the individual carboxyl carbon atoms of hen egg white lysozyme using carbon-13 NMR at natural abundance of ^{13}C (1.1%). Current and projected work includes the extension of these approaches to larger proteins, hemoglobin and protein hormones.

Objectives: 1) To obtain detailed information on the structure and function of proteins in solution. 2) To extend the applicability of the technique of high resolution NMR to macromolecules.

Major findings:

The four titrating histidine C2 proton resonances of ribonuclease have been unequivocally assigned to the four histidine residues in the primary sequence. This was accomplished by obtaining the rates of deuterium exchange of the C2 protons and comparing them with the rate of tritium exchange of the active site histidine residue 12 in ribonuclease S, under the same experimental conditions. This enabled a direct assignment of the resonance of this residue. By consideration of the results for carboxymethylated histidine derivatives it was possible to assign the resonance of the other active site histidine residine 119. These assignments are a reversal of those originally reported, and clear up some confusion in the literature on this subject. The assignment has important implications for the interpretation of NMR titration data on ribonuclease, which in turn has important implications for interpretations of the conformational transitions and mechanism of action of this enzyme.

Results for the histidine residues of ribonuclease S-protein (residues 21-124) indicate that the amino terminal portion (residues 1-20) are not necessary to produce a folded conformation in solution which resembles native ribonuclease. This appears to eliminate the possibility that has been suggested that proteins fold from their amino-terminal ends during protein synthesis *in vivo*.

Work on human, horse and sperm whale myoglobin has been extended to the deoxy and oxy forms. In all three proteins the reduction of the met-form to the deoxy form permits the observation of one additional titrating histidine resonance, and the oxygenation of the deoxy form permits the observation of a second additional titration curve with a low pK value. The origin of these titration curves is being investigated in terms of conformational changes in the protein or changes in the oxidation state of the iron atom in the heme prosthetic group. Other details of changes in pK values and electronic environment are interpreted in terms of the assignments of resonances made in previous work.

A derivative of cyctochrome c has been synthesized in which the two methionine methyl groups have been replaced with ^{13}C enriched methyls. One of these methionines, at position 80, is coordinated directly to the heme iron atom. By following the transitions of unfolding on alkali treatment and reduction at normal pH using carbon-13 NMR, it was possible to follow the fate of the resonances of these two methyl groups. This provides a tool for observing the specific environments of these residues, and the immediate environment of the heme.

Several of the carboxyl carbon atom resonances of hen egg white lysozyme have been resolved using carbon-13 nuclear magnetic resonance at 68 MHz. The change in chemical shift of the carboxyl carbon atom resonances as a function of pH has enabled the distinction of these resonances against the background of many non-titrating carboxyl group resonances. Several apparent microscopic ionization constants have been determined from the carboxyl group NMR titration curves. The resonances of the carboxyl side chains of aspartic and glutamic acids and the backbone carboxyl group in the free amino acids are known to be well resolved. This has allowed the identification of the resonances of one of the two glutamic acid carboxyl carbons, and possibly the terminal leucine carboxyl group, as well as several of the seven aspartic acid residues present in hen egg white lysozyme. In the presence of cobaltous ion, two carboxyl carbon atom resonances are found to shift selectively. It appears that one of these corresponds to the glutamic acid resonance and the other to an aspartic acid resonance. Therefore, we tentatively assign these resonances to the active site glutamic acid 35 and aspartic acid 52 residues, respectively. The pK_a value of 5.0 determined for the glutamic acid residue 35 is in the range expected for self-associated lysozyme. Our results indicate the possibility of observing directly a wide range of single functional groups of proteins in solution using NMR techniques. We have also carried out a theoretical analysis which indicates that contrary to prior treatments, higher magnetic fields are advantageous for carbon-13 NMR studies of proteins.

Proposed course of work:

a) Preliminary results indicate that to extend the observation of individually resolved histidine C2 proton resonances to the case of hemoglobin requires some additional approaches. This is mainly because of the higher molecular weight (the slower tumbling in solution results in broader resonances) and the larger number of histidine residues (19 separate sites, resulting in poorer resolution). The conditions for the selective deuteration of the histidine C2 protons have been determined, and the α and β chains of this partially deuterated Hb are being separated. They will then be recombined with normal material to observe only the resonances of the α or β chains separately in intact Hb.

b) The carboxyl carbon atom resonances of hen egg white lysozyme will be observed in the presence of oligosaccharide inhibitors. Since lysozyme aggregates at the high concentrations needed for the NMR experiments, we are studying the deaggregation induced by perturbants, including inhibitors. It would be valuable to know the effects of inhibitors and the aggregation process on the pK values of the carboxyl groups in the active site of lysozyme.

c) A goal of this project is to use carbon-13 NMR to investigate the structure of the sugar groups in glycoproteins and the effects of these groups on the conformation of the protein. Preliminary results on the ribonuclease A/B system indicates this is feasible and other systems for study are the DNase A/B/C/D system and protein hormones such as HCG.

Publications:

Matthews, C. R., Westmoreland, D., Hayes, M. B., and Cohen, J. S.: NMR Titration Curves of Histidine Ring Protons. VI. Effect of Temperature on Ribonuclease A. J. Biol. Chem., 250:7456-7460, 1975.

Hayes, M. B., Hagenmaier, H., and Cohen, J. S.: NMR Titration Curves of Histidine Ring Protons. VII. Human Metmyoglobin and the Effects of Azide on Human, Horse and Sperm Whale Metmyoglobins. J. Biol. Chem., 250:7461-7472, 1975.

Cohen, J. S., and Shindo, H.: NMR Titration Curves of Histidine Ring Protons. VIII. Conformational Change Affecting Three of the Histidine Residues of Ribonuclease. J. Biol. Chem., 250:8874-8881, 1975.

Shindo, H., Hayes, M. B., and Cohen, J. S.: NMR Titration Curves of Histidine Ring Protons. IX. A Direct Assignment of the Resonances of the Active Site Histidine Residues of Ribonuclease. J. Biol. Chem., in press.

Shindo, H., and Cohen, J. S.: NMR Titration Curves of Histidine Ring Protons. X. Ribonuclease S-Peptide and S-Protein. J. Biol. Chem., in press.

Shindo, H., and Cohen, J. S.: Observation of Individual Carboxyl Groups in Hen Egg White Lysozyme Using High Field Carbon-13 Nuclear Magnetic Resonance. Proc. Natl. Acad. Sci., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00033-03 RR

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Application of Stable Isotopes to Biochemical Problems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. S. Cohen	Research Chemist	RRB	NICHD
Other:	C. Niu	Visiting Fellow	RRB	NICHD
	S. Matsuura	Visiting Fellow	RRB	NICHD

COOPERATING UNITS (if any)

LAB/BRANCH
RRB

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	--

SUMMARY OF WORK (200 words or less - underline keywords)

The overall purpose is to develop applications of stable isotopes (^{13}C, ^{15}N, ^{18}O) as probes in biochemical systems with the application of appropriate techniques (NMR, GC-MS). In order to accomplish this objective the synthesis of molecules containing these isotopes enriched at selected positions is undertaken, notably synthesis of peptides containing ^{13}C and ^{15}N enriched amino acids. Carbon-13 or nitrogen-15 NMR will be used to study the structure and function in solution of such peptides, and proteins derived from them, utilizing the special advantages that such enrichment provides. Metabolic studies will be undertaken to investigate the use of stable isotopes as safe probes in human systems when a GC-MS instrument is available.

Objectives: 1) To synthesize compounds of biological interest containing isotopes of C, N and O at selected positions. Specifically to extend the use of the amino terminal peptide of ribonuclease as a model system for peptide hormone binding, by synthesizing derivatives containing selected ^{13}C and ^{15}N enriched amino acids. 2) To utilize the techniques of NMR and GC-MS to determine conformation in solution or metabolic pathways in organisms, respectively.

Major Findings:

As part of our program for the synthesis of peptides we have developed an improved method for the total deprotection of blocked products. Normally this is carried out in a Kel-F apparatus using gaseous HF. We have tested the reagent pyridine-poly-HF, and have found conditions which give complete de-protection of the standard blocked derivatives of the amino acids. This is a milder, less dangerous and less expensive procedure, and will probably become widely used.

The synthesis of the diketopiperazine cyclo Ala-Gly has been carried out to give 70% yield. The 1-15 amino terminal peptide of ribonuclease has been synthesized.

A stable acetyl intermediate of chymotrypsin catalysis has been prepared using p-nitrophenyl acetate and chymotrypsin at pH 5.1. The reagent has been prepared containing ^{13}C (90%) in the acetate carboxyl group. An assay system to determine chymotrypin at pH 5.1 has been developed.

Proposed Course of Project:

a) Uniformly ^{13}C enriched (85%) amino acids provided by Andrulis, Inc., are being purified and separated for use in peptide synthesis.

b) The dipeptides containing uniformly labelled ^{13}C-glycine and ^{15}N-alanine and the reverse ^{15}N-glycine-u.l.-^{13}C alanine are being synthesized. These will be cyclized to the diketopiperazines and the ^{13}C - ^{1}H, ^{13}C - ^{13}C and ^{13}C - ^{15}N couplings in the linear and cyclic form will be compared to see the effects of this gross conformational change. If selective effects are observed these will be important for the interpretation of effects in larger peptides.

c) The 1-15 peptide of ribonuclease will be synthesized containing two adjacent ^{13}C and ^{15}N enriched Ala residues in the region of the helix when bound to S-protein. Also, it will contain Asp at position 14 with a ^{13}C atom on the side chain carboxyl. This will provide a test both for backbone and sidechain conformational effects in a model system, particularly in view of our previous work which concluded that Asp-14 is involved in a pH-dependent conformational change.

d) The chymotrypin-acetyl intermediate will be synthesized with ^{13}C enriched acetate, and the NMR parameters including relaxation time, determined. These will enable an evaluation of the microdynamic and environmental changes on forming this active enzyme intermediate. The process of breakdown will also be followed. If successful, other derivatives in which specific electronic and steric effects could be expected will be synthesized.

e) On acquiring a GC-MS system this will be used for the determination of purity, amino acid content and isotopic analysis of the materials used in this project. It will also replace the need for amino acid analysis on routine analyzers. Applications of stable isotope materials to metabolic tracer work in organisms, including human systems, will also be investigated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00034-03 RR

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Binding Studies of Ligands or Hormones Using NMR

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. S. Cohen	Research Chemist	RRB	NICHD
Other:	W. Egan	Staff Fellow	RRB	NICHD

COOPERATING UNITS (if any)

Dr. M. B. Hayes, Cornell Medical School, N. Y.

LAB/BRANCH
RRB

SECTION

INSTITUTE AND LOCATION
NICHD, NIH

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2		

SUMMARY OF WORK (200 words or less - underline keywords)

To study the binding properties of small molecules, ligands and hormones, to protein sites in solution, using the dynamic characteristics of high resolution NMR.

PHS-6040
(12-75)

Objectives:

To determine the binding properties of small molecules to proteins and other macromolecules.

Major Findings:

The C-13 spin lattice relaxation behavior $(\equiv {}^1/T_1)$ for the carboxyl carbon atom of glycine has been investigated. It was found that T_1 varied significantly as a function of pH, showing distinct miminal values at approximately the carboxyl and amino pK_a values; this dependence of T_1 is attributed to the influence of trace paramagnetic metal ions which show a pH dependent binding. The sensitivity of T_1 to the presence of trace paramagnetic metal ions has significance for C-13 NMR binding studies.

Proposed Course of Study:

The binding of glycine to snake-venom L-amino acid oxidase will be studied by C-13 NMR; these results will be compared to a study previously carried out in this lab (Dr. M. B. Hayes) using proton NMR spectroscopy. Similar studies of aspartic acid binding to asparaginase are intended.

Publications:

Yeh, H. C., Kirk, K. L., Cohen, L. A. and Cohen, J. S.: ${}^{19}F$ and ${}^{1}H$ Nuclear Magnetic Resonance Studies of Ring-Fluorinated Imidazoles and Histidines, J. Chem. Soc., Perkin II, 928, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00035-04 RR

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structure-Function Relationship of Biologically Active Molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. Gross	Chief, Section on Molecular Structure,	RRB-NICHD
Other:	J. H. Brown	Research Chemist	RRB-NICHD
	H. C. Chen	Research Chemist	RRB-NICHD
	J. L. Morell	Research Chemist	RRB-NICHD
	L. J. Lin	Staff Fellow	RRB-NICHD
	P. Poisel	US-PHS Fellow	RRB-NICHD
	P. Fleckenstein	Visiting Fellow	RRB-NICHD
	S. Matsuura	Visiting Fellow	RRB-NICHD
	S. Nanno	Visiting Fellow	RRB-NICHD

COOPERATING UNITS (if any) National Cancer Institute, NIH, Bethesda, Maryland
G. Habermehl, University of Darmstadt, Darmstadt, Germany
P. Läuger, University of Constance, Constance, Germany
G. Jung, University of Tübingen, Tubingen, Germany

Reproduction Research Branch

Section on Molecular Structure

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.5	4.5	2.0

SUMMARY OF WORK (200 words or less - underline keywords)

The major objectives of the project are: (a) the structural analysis and synthesis of peptides and proteins with α,β-unsaturated amino acids, lanthionines, and lysinoalanine; (b) the correlation of the structural features of these molecules with their effect on neonatal and neoplastic tissue - retardation of intrauterine and tumor growth, inhibition of implantation, and induction of fetal resorption; (c) the synthesis of peptides corresponding to partial sequences of the carboxyl-terminal region of human chorionic gonadotropin for the development of immunoassays for the early detection and diagnostic evaluation of the hormone under normal and pathological conditions (d) the synthesis of analogues of the gramicidins A, B, and C for: iontransport studies across synthetic and biological membranes and further exploration of their established effect on neonatal and neoplastic tissue; (e) the synthesis of peptides agonistic or antagonistic to the release of fertility regulating hormones; (f) the application of DEHYDROALANINE RESINS to the solid phase synthesis of peptide amides; (g) the development of new techniques in (1) peptide synthesis - e.g. the protection of amino groups with γ-methylthiobutyric and, (2) amino acids analysis - column modification, detection, and sample application; (h) the development of nonenzymatic techniques for the fragmentation of peptides and proteins; (i) the isolation and characterization of lysosomes from developing tissue.

Project Description:

Objectives:

Analysis and synthesis of peptides: (a) with rarely seen structural features brought about by the presence of α,β-unsaturated and thioether amino acids, (b) representing partial sequences of hormones of the reproductive endocrine system for immunological exploration and application, (c) displaying hormone release or hormone release inhibiting properties, (d) affecting neonatal and neoplastic tissue, (e) showing ionophoric characteristics resulting in the transport of ions across artificial and biological membranes. Chemical, physical, and biological investigation of membrane-active peptides. Chemical modification of amino acids and development of nonenzymatic reactions for the fragmentation of peptides and proteins. Isolation and characterization of lysosomes from developing tissue. Development of new approaches to the synthesis of physiologically significant peptides. Design, development, and implementation of new techniques and instrumentation in amino acid analysis and peptide synthesis.

Methods Employed:

Thermophylic proteases, new exopeptidases, and concurrently developed nonenzymatic methods for the structural elucidation of peptides with uncommon amino acids. Dehydroalanine resins in the solid phase synthesis of peptide amides. Alkylated dehydroalanine amides for the incorporation of amides in endo-positions. Novel and selectively removable protecting groups in peptide synthesis. Stereochemically controlled synthesis of amino acids with multiple centers of asymmetry. Advanced separation and analytical techniques; specifically developed sample application and data acquisition in amino acid analysis; counter double current distribution. Circular dichroism (CD); mass spectrometry; X-ray analysis in structural studies; carrier-free electrophoresis.

Major Findings:

Utilization of DEHYDROALANINE RESINS in solid phase synthesis in combination with β-eliminable amino acids, such as S-alkyl cysteine or O-substituted serine, offers the alternative of incorporating free carboxyl groups or the amide function in amino acid residues in the carboxyl-terminal position, a feature important for the synthesis of partial hormone sequences and their immunological exploration. Synthetic peptides corresponding to partial sequences of the carboxyl-terminal region of human chorionic gonadotrophin (hCG) bind to antibodies raised against the tryptic fragment consisting of the last 23 residues of hCG (cf. Z01 HD 00027-04 RR). Structural information from fragments obtained after proteolytic digestion with and/or without desulfurization removes existing ambiguities in the structure of DURAMYCIN in favor of this sequence: H_2N-ALA(1)-LYS-GLU-ALA(4)-ALA(5)-ABA(6; aminobutyric acid)-PHE-GLY-PRO-PHE-ALA(11)-PHE-VAL-ALA(14)-HO-ASP-GLY-ASP-ABA(18)-LYS(19)-COOH. Crosslinkages in the molecule are assigned as follows: sulfide bridges, LAN 4-14; β-CH_3-LAN, 1-6 and 11-18; imino bridge of LYSINOALANINE, 5-19. The sterospecific synthesis of β-methyl-D-threo-cysteine, a key purcursor of β-methyl-lanthionine for the synthesis of fetal resorption inducing fragments of nisin, was initiated with D-threonine and successfully carried through several stages of inversion in configuration before establishing final stereochemical homogeneity. The utility of the γ-methylthiobutyryl group as protection of ω-amino groups was demonstrated by the solid phase as well as the solution synthesis of pyroglutamyllysylglycine amide, the first low molecular weight peptide showing antagonist activity towards luteinizing hormone. The fragmentation of peptides and proteins by cleavage of the S-methylcysteinyl peptide bond by cyanogen bromide may be suppressed in favor of cleaving the methionyl peptide bond by the same agent, thus offering another discriminatory feature of the nonenzymatic order. Solid phase synthesis of gramicidin analogues for ion transport and fetal resorption studies. Lysinoalanine, the natural constituent of CINNAMYCIN and DURAMYCIN is formed upon the alkaline treatment of soya and whey proteins (traces of lanthionine are also seen in the latter case). Lysinoalanine formation in alkali treated nisin occurs intra- and intermolecularly. Total number of amino acid analyses on three instruments of the Section: 880; 81 of these for other laboratories of the Institute and at NIH.

Significance to Biomedical Research and the Program of the Institute

The analysis of peptides with unusual amino acids provides the structural information necessary for the understanding of their mode of action on endocrine functions. Nisin, fragments of nisin, and all analogues of the gramicidins A, B, and C thus far tested, induce fetal resorption in the rabbit and/or the rat. The addition of essential sulfhydryl groups across the double bonds of α,β-unsaturated amino acids may be responsible for the effect seen. Ionophoric properties are not to be ruled out for nisin and nisin fragments; they play, in all likelihood, an important role when the gramicidins act on intrauterine tissue. In another reaction, α,β-unsaturated amino acids are capable of adding water across their double bond and the conversion to amide and ketoacid, two entities of most widely recognized physiological significance. The carboxyl-terminal amide function is common to all structurally established hormone releasing factors and seen in many instances of synthetic peptides possessing hormone release inhibiting potential. The development of DEHYDROALANINE RESINS and their application to peptide amide synthesis is the direct outgrowth of the discovery of dehydroalanine and dehydrobutyrine in nisin and subtilin by this laboratory. Today this method of peptide amide synthesis is in continued use to provide thyrotropin releasing hormone for clinical use. The unique alternating pattern in configurations at the α-carbon atoms of the amino acids of the gramicidins A, B, and C enables these molecules to form specific types of helices which - in the form of head to head dimers - are capable of spanning lipid bilayers (and possibly biomembers), and transporting ions via channel mechanisms. The study of the mechanism of action of the effect of gramicidin analogs on intrauterine tissue will be greatly facilitated by the availability of synthetic variants, even more so, by the direct synthetic incorporation of radiolabel. Access to synthetic partial structures of low molecular weight peptides corresponding in sequence to, or constituting tolerable alterations of segments of protein hormones, such as human chorionic gonadotrophin, offer great potential as diagnostic tools for the early immunological detection of these compounds in physiological fluids derived from normal and/or abnormal states.

Proposed Course of Project:

Synthesis of peptides comprising partial sequences of the last 28 amino acids of carboxyl terminal region of human chorionic gonadotropin. Chemical modification and/or incorporation of functions for the ready analytical detection of these peptides in immunoassays for chorionic gonadotropin in samples from normal physiological and/or pathological conditions (cf. the ectopic production of gonadotrophin in tumor tissue). Synthesis of analogues of gramicidins A, B, and C - incorporation of radiolabel into gramicidins for autoradiographic studies. Synthesis of fragments of nisin - exchange of amino acids in and incorporation of radiolabel into those peptides. Synthesis of peptide agonists and antagonists for hormone release with particular emphasis on the pursuit of low molecular weight peptide amides inhibiting the release of luteinizing hormone. Development and improvement of techniques of peptide synthesis, such as the introduction of new protecting groups for amino acids based on the chemistry of thioether and α,β-unsaturated amino acids. Development of nonenzymatic methods (a) generally applicable to the fragmentation of peptides and proteins; (b) designed for the specific requirements of the elucidation of unique structural features, such as those seen in DURAMYCIN and CINNAMYCIN. Correlation of the closely related structural features (α,β-unsaturated amino acids, lanthionines, lysinoalanine) in peptides (nisin, subtilin, cinnamycin, and duramycin) of strikingly different origin followed by an analysis of phylogenetic aspects. Purification and characterization of peptides of natural origin and chemical synthesis of peptides to investigate their intrauterine growth inhibitory properties. Broadly based screening for compounds capable of introducing fetal resorption. Spectroscopic investigation (nuclear magnetic resonance with different nuclei; circular dichroism) of nisin fragments and the gramicidins A, B, and C, and the interaction of these peptides with synthetic membranes of known composition and well characterized biomembranes. Iontransport and conductance studies with the transmembrane channel forming peptides gramicidin A, B, and C and their respective analogs. Isolation and characterization of subcellular constituents, primarily lysosomes from developing tissue. Improvement in the design and construction of amino acid analyzers and sample applicators used in amino acid analysis.

2. Publications

Gross, E., Noda, K., and Matsuura, S.: The utility of α,β-unsaturated amino acids in peptide synthesis. In Wolman, Y. (Ed.): Peptides 1974 New York, New York, John Wiley and Sons, 1975, pp. 403-414.

Gross, E. and Matsuura, S.: α,β-Unsaturated and thioether amino acids in peptide synthesis. In Walter, R. and Meienhofer, J. (Eds.): Chemistry and Biology of Peptides. Ann Arbor, Michigan, Ann Arbor Publishers, 1975, pp. 351-358.

Gross, E.: Subtilin and nisin: The chemistry and biology of peptides with α,β-unsaturated amino acids. In Walter, R. and Meienhofer, J. (Eds.): Chemistry and Biology of Peptides, Ann Arbor, Michigan, 1975, Ann Arbor Publishers, pp. 31-42.

Bamberg, E., Läuger, P., Gross, E. and Noda, K.: Single channel parameters of gramicidins A, B, and C. Biochimica et Biophysica Acta 419:223-228, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00036-01 RR

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Carbon-13 NMR Studies of Cell Wall Polysaccharides; Structure and Microdynamics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William M. Egan	Staff Fellow	RRB/NICHD
	Jack S. Cohen	Research Chemist	RRB/NICHD

COOPERATING UNITS (if any)

D. Liu, Bureau of Biologics, Food and Drug Administration, Bethesda, Md.

LAB/BRANCH
RRB

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

SUMMARY OF WORK (200 words or less - underline keywords)

It is the goal of this project to determine the structure and microdynamic behavior of antigenic bacterial cell wall polysaccharides, primarily through the application of carbon-13 NMR spectroscopy. Under current investigation are: 1) N. Meningitidis serogroups B and C capsular polysaccharides and the immunologically cross-reactive (to serogroup C) E.Coli. strains, Bos-12, MT-411, and NT 67; 2) H.Influenza, type b, polysaccharides and their immunologically cross-reactive E.Coli strains, Easter, 10,89,BCH, and Kf 147; 3) The Pneumococcus C polysaccharides.

PHS-6040

Project Description

Objectives:

To determine the structure and microdynamic behavior of antigenic bacterial cell wall polysaccharides, primarily through the application of carbon-13 NMR spectroscopy.

Major Findings:

E. Coli, strain Bos-12 (048; K?; NM), is an enteric bacterium unassociated with known human disease, but which cross-reacts with meningococcal Group C antibodies (Gotschlich et al, J. Exp. Med. 129; 1349, 1969). We have furnished evidence, based on carbon-13 NMR spectroscopy, for the structure of this cross-reacting E. Coli polysaccharide as an α-2,8/α-2,9 linked heteropolymer of sialic acid; high resolution proton NMR spectroscopy has demonstrated an approximately 1:1 ratio of α-2,8 and α-2,9 linkages. Subsequent NMR studies on additional cross-reacting (to meningococcal Group C antigens) E. Coli strains (MT-411 and NT-67) revealed an identity of polysaccharide structure.

Proposed Course of Project:

1. Carbon-13 NMR studies have shown that the Group C meningococcal and Bos-12 polysaccharides differ in structure (the Group C polysaccharide is a pure α-2,9 linked polymer of sialic acid while Bos-12 is an α-2,8/α-2,9 linked hetereopolymer of sialic acid); emphasis will now be placed on comparing the microdynamic behavior of these two antigens in an effort to further elucidate the cause of their cross-reactivity.

2. Initial studies have revealed the identity of several strains of H. Influenza as ribose ribitol polymers. Attempts will be made to determine the structure of the capsular polysaccharides of cross-reacting E. Coli strains (Easter, 10, 89, BCH, and Kf 147).

3. Studies to elucidate the structure of the complex Pneumococcus C cell wall are in progress.

Publications:

W. Egan, T.-Y. Liu, J. S. Cohen and J. B. Robbins, in "Proceedings of The Second International Conference on Stable Isotopes," in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00037-01 RR

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Conformational Behavior and Chemical Stability of Selected Anticancer Drugs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William M. Egan	Staff Fellow	RRB/NICHD
Other:	Jack S. Cohen	Research Chemist	RRB/NICHD
	Girjesh Govil	Visiting Scientist	LCP/NIAMDD

COOPERATING UNITS (if any)

Dr. Gerald Zon, Catholic University of America, Washington, D. C.

LAB/BRANCH
RRB

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to elucidate the interrelationship between anticancer activity and conformational preference of selected alkylating agents; consideration is also given to several aspects of chemical stability. Under current investigation are the P-N alkylating agents, cyclophosphamide and thiotepa, and their derivatives. The stability and conformational behavior of these agents are being probed by high-resolution NMR spectroscopy and molecular orbital calculations. It is hoped that insights gained through these studies will aid in the design of improved anti-tumor agents.

Objectives:

1. To determine the connection between conformational preference and anticancer activity in P-N alkylating agents.

2. To use high resolution NMR spectroscopy to study the chemical stability of P-N alkylating agents and to determine the structure of their breakdown products.

Major Findings:

In connection with determining the enzymatic stereospecificity for in vivo activation (C-4 hydroxylation) of the anticancer drug cyclophosphamide (CPA) it became necessary to evaluate the dynamic conformational properties of the six-membered heterocyclic ring. Variable temperature H-1 and C-13 NMR studies indicated that ring-reversal of the six-membered ring was rapid on the NMR time scale and that both chair forms were significantly populated.

To better define the cyclophosphamide conformational surface, molecular orbital calculations were performed. These calculations showed that the two chair forms were comparable in energy (to within 0.5 kcal/mole), but only ca. 4-5 kcal/mole more stable than the boat form having the mustard functionality in the exo position; the boat form with an endo mustard is ca. 20 kcal/mole less stable than the exo conformer.

The stability of the anticancer agent, 1,1',1" phosphinothioylidynetrisaziridine (thiotepa) in acidic and saline media has been investigated by H-1 NMR. In acidic solutions, with or without added NaCl, thiotepa was shown to be quickly degraded via acid catalyzed P-N bond cleavage and/or ring opening; however, with 0.5 equivalents of acid, ca. 25% of the thiotepa remains. Additionally, thiotepa was shown to be relatively stable in saline; after two days at room temperature no decomposition was observed; at 55°, however, thiotepa undergoes a gradual ($t_{1/2} \approx 3$ day) decomposition. Our findings regarding the reactions and stability of thiotepa contradict previous reports (Mellet and Woods, Can. Res., 20: 524, 1960; Benckhuijsen, Biochem. Phar., 17: 55, 1968) based on indirect chemical evidence (e.g., functional group tests).

Proposed Course of Study:

1. Attempts to define the complex of reaction products and pathways available to cyclophosphamide and thiotepa, via hydrolysis and thermolysis, will be made; similar studies will also be made on a number of derivatives.

2. Further studies on the conformational mobility of cyclophosphamide derivatives will be made in order to better define the connection between CPA activation and molecular conformation.

Publications:

1. Egan, W. and Zon, G.: High Resolution ^1H and ^{13}C NMR Investigations of Cyclophosphamide Conformational Stability, Tetrahedron Letters, in press.

2. Zon, G., Egan, W. and Stokes, J. B.: Observations of 1,1',1"-phosphinothioylidynetrisaziridine (thiotepa) in Acidic and Saline Media. A ^1H NMR Study, Biochem. Pharm., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00038-01 RR

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Relaxation Time Studies of Water in Normal and Cancerous Cell

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. S. Cohen	Research Chemist	RRB	NICHD
	E. D. Becker	Chief	LCP	NIAMDD
Other:	R. Ader	Visiting Fellow	LCP	NIAMDD

COOPERATING UNITS (if any)
E. B. Thompson, LB, NCI.

LAB/BRANCH
RRB

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0		

SUMMARY OF WORK (200 words or less - underline keywords)

To determine under carefully controlled conditions if there is a difference in the relaxation times (T_1, T_2) of bulk water in normal and transformed cells grown in culture. If a significant difference is observed, to determine the origin of the effect.

PHS-6040
(12-75)

Objectives:

1. To test the proposition that the relaxation times of bulk water in cell systems is dependent on whether the cells are normal or cancerous.
2. To determine characteristic values of relaxation time per cell or per unit volume of packed cells.
3. To elucidate the origin of any difference in relaxation times between different cell types.

Instrumental Modifications:

The following instrumental modifications had to be made before experimental work could be started.

1. The "Spin-Lock" pulsed spectrometer was interfaced to the JEOL magnet system. This consisted among other things in constructing a flux stabilizer unit and a linear sweep field unit, to provide additional stabilization and control of the magnetic field.
2. A sample probe based on the original JEOL probe head was constructed and was equipped with variable temperature accessories. For improving magnetic field homogenity in T_2 measurements, a shim system was added. To enable the measurement of diffusion coefficient gradients, coils were added.
3. To speed up measurements and increase accuracy, a digital data acquisition system was built and interfaced.

Major Findings:

With the existing instrumentation the following preliminary studies have been carried out.

Relaxation times T_1 and T_2 were measured under the same conditions on several cell lines grown in culture by Dr. Thompson.

The best experimental conditions for carrying out these measurements on cells were investigated. Centrifugation at ca. 250 g was used to obtain a suitably packed sample. It was found that the presence of an appreciable amount of supernatant in the sample placed within the receiver coil resulted in longer T_1 values. This is due to the fact that the T_1 of the aqueous buffer used for washing the cells is longer than of cellular water.

In addition the cells were counted to ensure the total number of cells were known in the sample volume, which was usually enough to fill the receiver coil. It was found that up to five hours after the experiment was started there was only small change (no more than 10%) in T_1 values, and that trypsinization was the most efficient means of removing the cells from the growth flask. Approximately 4 - 7% of cells were found dead, as based on the trypan blue method, performed before the NMR measurements were started. About 20% of the cells were dead after all the NMR measurements had been completed within ca. 5 hours. Those results suggested the use of a larger number of smaller flasks for the large amount of cells needed for those studies to obtain fewer dead cells.

Two comparative studies were carried out between normal and viral transformed cancerous cells

The cell lines studied were NRK/KNRK and SRNRK; KA31/B3T3. The results of these studies were different. In the former case there was very little change (ca 4%) between the relaxation times for the normal and transformed cells, which may be considered within experimental error, while for the latter cell type a difference of 40% was obtained.

Relaxation time measurements on diseased cells which were submitted to drug treatment showed differences in T_1 values of 8%, and 20% in the latter case of leukemic cells.

Proposed course of project:

Before any conclusions can be drawn from these studies, more comparative data must be obtained from normal vs cancerous cells, using NMR. In addition total water content as well as intracellular and extracellular water content should be determined together with paramagnetic ion content. These findings should be related to the relaxation times measurements.

Finally a suitable physical picture should be arrived at to explain the results obtained.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00039-01 RR

PERIOD COVERED

July 1, 1975 to September 30, 1975

TITLE OF PROJECT (80 characters or less)

Cleavage Patterns in Mammalian Eggs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: B. J. Gulyas Senior Investigator RR NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Reproduction Research Branch

SECTION

Section on Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

SUMMARY OF WORK (200 words or less - underline keywords)

It was the purpose of this project to reexamine the pattern of cleavage in mammalian eggs using semi-cinematographic techniques. The evidence shows a rotational cleavage pattern in mammalian eggs, which is distinct from previously reported cleavage patterns in other animals.

PHS-6040
(12-75)

The pattern of cleavage, characteristic of a particular species, emerges from the positioning of the cleavage furrows with respect to each other. The positioning of cleavage furrows in various vertebrate and invertebrate animals have been studied extensively by classical embryologists. However, only a few observations are available on the pattern of cleavage in mammalian eggs. Some of these observations suggest that mammalian eggs might differ in their pattern of cleavage from lower vertebrates and invertebrates. The purpose of this project was to examine the pattern of cleavage in rabbit, mouse and rat eggs. A Dvorak-Stotler chamber was used to culture the eggs. Individual or groups of fertilized eggs were photographed at various intervals through cleavage. It was demonstrated that the pattern of cleavage in the rabbit differs from other animals, such as sea urchin, because the blastomeres at the 4-cell stage are arranged crosswise in the former, while they are situated next to each other in the latter. The crosswise arrangement of the blastomeres was a consequence of a 90^{o} rotation of the polar axis in one hemisphere of the egg. These observations indicate that the pattern of cleavage in the rabbit departs from the classic patterns shown by lower vertebrates and invertebrates. Closer examination of recent photomicrographs of mink, pig, baboon, monkey, and human eggs revealed a crosswise arrangement of the blastomeres at the 4-cell stage. On the basis of evidence we conclude that in most eutherian mammals rotation of the polar axis occurs in one of the two blastomeres during second cleavage. This project has come to completion during 1975.

Publications:

Gulyas, B.J.: A reexamination of cleavage patterns in eutherian mammalian eggs: Rotation of blastomere pairs during second cleavage in the rabbit. J. Exp. Zool., 193: 235-248, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00040-01 RR

PERIOD COVERED

June 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Models of Hormone-Receptor Interaction and Endocrine Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. Rodbard, M.D., Senior Investigator, RRB, NICHD, NIH
PI:	G. Aurbach, M. D., Chief, Metabolic Res. Br., NIAMDD, NIH
Other:	E. M. Brown, M.D., Clinical Associate, MRB, NIAMDD, NIH

COOPERATING UNITS (if any)

University of Manitoba, Manitoba, Canada; Department of Pediatrics, University of Pennsylvania; Department of Physiology, Laboratory of Human Reproduction, Reproductive Biology, Harvard University

LAB/BRANCH

Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.7	.5	.2

SUMMARY OF WORK (200 words or less - underline keywords)

Mathematical models have been developed to described hormone-receptor interactions in complex cases involving heterogeneity of hormone and receptor, positive and/or negative cooperativity, and failure to achieve equilibrium or steady-state conditions. By use of computer simulations and curve-fitting techniques, we have derived rate constants and equilibrium constants for binding of human and bovine growth hormones, and of ovine prolactin to rat hepatocytes under a variety of physiological conditions; binding of catecholamines and their synthetic analogs to erythrocytes and erythrocyte membranes; and binding of hCG to rat Leydig cells. A new method has been developed which can, at least in principle, permit calculation of affinity constants, binding capacities, and allow construction of Scatchard plots, without the use of radioisotopes. The effect of factors such as threshholds, spare receptors, and cooperativity on the shape of dose response curves has been analyzed by simulation techniques.

Objectives:

This project has a goal of developing mathematical, statistical, and conceptual models of hormone-receptor interactions and the coupling of hormone binding to physiological actions. These models deal with the protein and polypeptide hormones, but are also applicable to steroids, drugs and neurotransmitters. In addition to mathematical modeling of hormone-receptor and receptor-effector systems, we have developed mathematical models for several other areas of endocrinology. These include mathematical modeling of the hormonal changes which occur during the normal menstrual cycle. The model developed by Bogumil, Vande Wiele et al., has been revised, expanded, and improved considerably. Also, mathematical modeling of the effects of chemotherapy in patients with gestational trophoblastic disease has begun, and shows considerable promise.

Methods Employed:

Techniques of mathematical modeling, including computer simulation and curve-fitting techniques.

Major Findings:

A new technique has been developed (in collaboration with W. Moyle) which, in principle, allows for the estimation of the Scatchard plot, affinity constants, and binding capacities of a target tissue, without the use of radioisotopes. This is obtained by the simple method of obtaining "steady-state" dose response curves for the hormone, as one systematically varies the concentration of cells. By assuming that the response (as percent of maximal response) of the tissue is a function of the free hormone concentration, one can calculate by fairly straightforward techniques, the bound to free ratio for the hormone. The statistical treatment of these data is particularly complex. It was demonstrated that rat hepatocytes contain both a "somatogenic" binding site and a "lactogenic" binding site. Livers from male rats contained only the somatogenic site. Treatment of males with estradiol results in appearance of the "lactogenic" site. Hypophysectomy blocks this response to estradiol; presumably prolactin is responsible for induction of its own receptors. The relative affinities of a wide number of catecholamine agonists and antagonists have been evaluated in the turkey erythrocyte membrane system and in intact cells. The constants describing the association and dissociation rates, the equilibrium constants and binding capacities have been developed. An excellent correlation was found between affinity and ability to activate adenylate cyclase.

The effect of extraneous, soluble binding proteins on the interactions of hGH with rat hepatocyte membranes has been analyzed from a theoretical standpoint (with H. Friesen).

Effect of threshholds, spare receptors, and nonlinear relationships between receptor occupancy and hormonal response on the shape of the observable dose response curve have been analyzed in detail.

Significance to Biomedical Research and the Program of the Institute:

Much current research interest centers around the binding of hormones, drugs and neurotransmitters to their naturally occurring receptors. The models which have been developed here should be generally applicable both to endocrine and non-endocrine systems.

Proposed Course:

Study will continue to evolve in collaboration with several experimentalists here at NIH and throughout the country. The models will be progressively refined and used as guides to experimentation; as new experimental results become available, the models will be refined further. In the process, a series of computer programs have been developed for progressively "routine" use for analysis of binding systems and as an aid in model building. The experimental systems presently being used, involving receptors for growth hormone, prolactin, hCG, and catecholamines will continue to be used. It is anticipated that additional systems, involving steroid receptors, hypothalamic hormones, and possibly neurotransmitters will be added.

We shall continue to develop mathematical models to attempt to describe the course of gestational trophoblastic disease, including consideration of the doubling time of the tumor, the ED50 for the chemotherapeutic agents, the percent of cells which are susceptible to the agent, and related parameters. Also, the effects of the chemotherapeutic agents on the bone marrow will be evaluated by a variety of mathematical and statistical models. These models will be developed with the goal of providing useful information to the clinician, to aid in evaluating how much chemotherapy to give and when, in order to obtain maximal beneficial response with minimal bone marrow toxicity.

Publications:

Brown, E. M., Fedak, S. A., Woodard, C. J., Aurbach, G. C., and Rodbard, D., Beta Receptor Interactions: Direct Comparison of Receptor Interactions and Biological Activity, J. Biol. Chem., in press.

Ranke, M. B., Stanley, C. A., Rodbard, D., Baker, L., Bongiovanni, A. M., and Parks, J. S., Sex Differences in the Binding of Human Growth Hormone to Isolated Rat Heptocytes, Proceedings of the National Academy of Sciences, U.S., in press.

Note: Several other publications dealing with this work have been submitted or are in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00041-01 RR

PERIOD COVERED

July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cardiovascular Effects of Thyroid, Catecholamine Hormones and Cardiotoxic Drugs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	D. Rodbard, M. D., Senior Investigator, RRB, NICHD
Other	F. A. Greco, M. D., Clinical Associate, Radiation Oncology Br., NCI

COOPERATING UNITS (if any)

Department of Medicine, UCLA

LAB/BRANCH

Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of thyroid hormone and beta adrenergic agonists on target tissues may be evaluated by noninvasive monitoring of cardiovascular function. New techniques have been developed and evaluated for this purpose. The QK$_d$ interval, i.e. the interval between the onset of ventricular depolarization and the arrival of the pulse wave at the brachial artery, provides a rapid, innocuous, precise, reproducible noninvasive measurement for evaluation of thyroid, cardiovascular, or catecholamine status. This has been used for improved diagnosis and monitoring of therapy for patients with thyrotoxicosis, hypothyroidism, pheochromocytoma, and in the titration of effects of beta-adrenergic blockers such as propranolol. This method for noninvasive monitoring of cardiovascular patients has been applied in a prospective manner to evaluate patients receiving adriamycin chemotherapy, in an attempt to monitor cardiovascular toxicity. Preliminary results indicate a very high incidence of adriamycin cardiotoxicity and may make it possible to predict which patients develop overt congestive heart failure.

PHS-6040

Objectives:

Develop and evaluate methods for noninvasive monitoring of thyroid status, catecholamine stimulation or blockade, and myocardial contractility in patients with drug-induced or other cardiomyopathies.

Methods Employed:

A new method has been developed called "Sphygmo-Recording". The electrocardiogram, phonoarteriogram, and the pressure from the blood pressure cuff are fed into a specially designed electronic apparatus which automatically displays the calibrated contour of the arterial pressure wave on the phase of a storage oscilloscope. This is then photographed by Polaroid camera. This electronic instrumentation has also been utilized for improved recording of heart sounds, carotid sounds, thyroid bruits and a variety of plethysmographic signals.

Major Findings:

In patients with thyrotoxicosis there is a significant shortening of the interval between the QRS complex and the arrival of the pulsewave at the brachial artery (to 100 msec). This result is highly statistically significant and is of sufficient utility to be of diagnostic value in individual patients. In hypothyroidism, there is a marked prolongation of the QK_d interval (to as much as 300 msec), and again this technique may be utilized to establish a presumptive diagnosis in the absence of any other tests, and even in the absence of a clinical picture of hypothyroidism. This has been useful in evaluating patients with secondary amenorrhea. In patients with pheochromocytoma, there may be a marked shortening of the QK_d interval and an increase in the slope of the arterial pressure wave upstroke (dP/dt). The shortening of the QK_d interval appears to correlate with the level of either endogenous or exogenous catecholamines. QK_d is shortened during infusions of the beta adrenergic agonist, isoproterenol. Propranolol results in a marked prolongation of the QK_d interval. Patients receiving adriamycin chemotherapy show a significant (greater than 20 msec change) increase in the QK_d interval appearing 2-3 weeks after administration of the drug in a variety of dosage regimens, alone or in combination with other drugs or radiation therapy. If the drug is given only every 7 weeks, then the QK_d usually returns to the normal baseline level prior to administration of the next course of chemotherapy. Patients receiving the drug every 3 weeks appear to have a cumulative toxicity, wherein the QK_d interval remains persistently elevated (with a mean of 50 msec) on the first day of the second and third courses of chemotherapy. A combination of known high dose levels of adriamycin, in patients who showed a persistent elevation of QK_d interval, was correlated with a very high incidence of overt congestive failure. The Sphygmo-Recorder was used to document the presence of third or fourth heartsounds in these patients.

Significance:

Despite the proclivity of chemical tests, particularly radioimmunoassays for thyroid hormones, there is no generally available reliable measure of thyroid effects on the target tissues for routine clinical use. The present study provides one such measure of thyroid effects on the end organ. In addition, it is useful in the monitoring of therapy for pheochromocytoma and for beta adrenergic stimuli and blockers. This method is useful in evaluating the cardiotoxicity of the important anti-tumor agents, and the newly developed analogues, which also show myocardial toxicity.

Proposed Course:

The use of this technique for monitoring the thyroid status is now sufficiently well established that it begins to shift from a research project to a matter of routine clinical service. However, some interesting research projects remain, particularly in reference to the interaction between thyroid hormones and catecholamines. We intend to study the effect of propranolol administered to patients showing thyrotoxicosis, to evaluate the role of adrenergic pathways in the mechanism of action of thyroid hormones. Also, the utility of this measurement in children remains to be fully established and validated; correction factors need to be introduced for the height of the patient. The use of this technqiue for monitoring of patients with drug induced cardiomyopathies will be an ongoing project in collaboration with the Radiation Medicine Branch of the National Cancer Institute.

Publications.

Young, R. T., Van Herle, A. J., and Rodbard, D., Improved Diagnosis and Management of Hyper- and Hypothyroidism by Timing of the Arterial Sounds, J. Clin. Endocrinol. Metab. 42: 330-340, 1976.

Note: Several other articles are in preparation or submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00042-01 RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Mathematical and Statistical Theory of Polyacrylamide Gel Electrophoresis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI David Rodbard, Senior Investigator, RRB, NICHD, NIH
Other Andreas Chrambach, Senior Investigator, RRB, NICHD, NIH
Tohru Murakami, Visiting Fellow, RRB, NICHD, NIH
George H. Weiss, Chief, Phys. Sci. Lab., DCRT, NIH

COOPERATING UNITS (if any)
Max Planck Insitute, Gottingen, West Germany
Massachusetts Institute of Technology, Cambridge, Massachusetts

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0

SUMMARY OF WORK (200 words or less - underline keywords)

The mathematical and statistical principles of polyacrylamide gel electrophoresis, SDS polyacrylamide gel electrophoresis, isoelectric focusing, gel filtration and related physical-chemical techniques have been developed. This is to permit optimization of conditions for fractionation of protein and polypeptide hormones, hormone receptors and other macromolecules. Methods have been developed to permit improved statistical estimation of molecular weights derived from these techniques. This project also studies the electrophoresis and sedimentation properties of cells in sucrose gradients and optimization of two-dimensional macromolecular mapping.

PHS-6040
(12-75)

Objectives:

Improvement of the performance of physical-chemical techniques used for the analysis and purification of protein and polypeptide hormones.

Methods Employed:

Polyacrylamide gel electrophoresis, gel filtration, sucrose density sedimentation, sucrose density electrophoresis of cells and proteins, isoelectric focusing with continuous scanning and computation of molecular parameters.

Major Findings:

Improved methods have been developed for calculation of molecular weights from polyacrylamide gel electrophoresis, SDS-PAGE, gel filtration, and related techniques. It is found that the popular techniques for estimation of molecular weight, Stoke's radius, diffusion coefficient and related techniques are often invalid or subject to errors which are several times larger than those commonly appreciated by practitioners of the methods. Several sources of systematic bias have been identified. Computer programs for the proper analysis of these data have been developed and applied to several systems including studies of rhodopsin, protein kinases, glycoprotein receptors of liver. Studies on fractionation and characterization of the uterine-estradiol receptor have been undertaken.

Significance to Biomedical Research and Program of the Institute:

The use of SDS-PAGE and gel filtration for molecular weight determinations have been extremely popular; yet virtually none of the users of these methods have utilized appropriate statistical methods or mathematical models for the analysis of their results. The techniques developed in this study permit proper application of nonlinear weighted least squares regression methods, permit more reliable estimation of molecular weight, or sensitive detection of nonlinearity or invalidity of results, and reliable estimation as to the precision of a molecular weight, molecular radius and other parameters being measured.

Proposed Course:

The project involving both theoretical and experimental aspects will continue in the attempt to develop more useful methods for characterization of protein and polypeptide hormones. Use of continuous scanning of electrophoresis columns to permit estimation of the diffusion coefficient, molecular radius, and microheterogeneity of size or charge, is to continue; together with the corresponding theoretical studies necessary for the automated computer analysis of results.

Publications.

Frank, R. N. and Rodbard, D., Precision of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis for the Molecular Estimation of a Membrane Glycoprotein: Studies on Bovine Rhodopsin. Archives Biochem. Biophys. 171: 1-13, 1975.

Catsimpoolas, N., Griffith, A. L., Williams, J. M., Chrambach, A., and Rodbard, D., Electrophoresis with Continuous Scanning Densitometry: Separation of Cells in the Density Gradient. Anal. Biochem. 69: 372-384, 1975.

Rogol, A., Ben-David, M., Sheats, R., Rodbard, D., and Chrambach, A., Charge Properties of Human Pituitary and Amniotic Fluid Prolactins, Endocrinological Res. Comm. 2: 379-402, 1975.

Chidakel, D. E., Baumann, G., Rodbard, D., and Chrambach, A., A Simple Electronic Device for the Measurement of Band Migration Distances in Polyacrylamide Gels, Anal. Biochem. 66: 540, 1975.

Chrambach, A., Jovin, T., Svendsen, E. J., and Rodbard, D., Apparatus and Procedures for Analytical and Preparative Polyacrylamide Gel Electrophoresis Using an Objectively Defined Fractionation Route, in Methods of Protein Separation, Vol. 2, N. Catsimpoolas (ed.), Plenum Press, New York, in press.

Rodbard, D., Determination of Molecular Weight and Radius by Gel Filtration, and Polyacrylamide Gel Electrophoresis (PAGE) and PAGE in Sodium Dodecyl Sulfate. 1. Mathematical Principles, in Methods of Protein Separation, Vol. 2., N. Catsimpoolas (ed.), Plenum Press, New York, in press.

Rodbard, D., Determination of Molecular Weight by Gel Filtration, Polyacrylamide Gel Electrophoresis (PAGE) and SDS-PAGE: Statistical and Computational Considerations, in Methods of Protein Separation, Vol. 2, N. Catsimpoolas (ed.), Plenum Press, New York, in press.

Weiss, G. H., and Rodbard, D., Measures of Resolution for Multicomponent Systems in One and Two Dimensions with Application to Pore Gradient Electrophoresis, Separation Sci., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00043-01 RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Mathematics and Statistics of Radioligand Assays

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI D. Rodbard, M.D., Senior Investigator, RRB, NICHD, NIH
Other S. McLean, Clin. Chem. Serv., Clin. Path. Dept., CC, NIH
M. Yanagishita, Visiting Fellow, RRB, NICHD, NIH

COOPERATING UNITS (if any)
Food and Drug Administration
Walter Reed Army Institute of Medical Research
Stanford University

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	1.25	1.

SUMMARY OF WORK (200 words or less - underline keywords)

The mathematical and statistical theories of radioimmunoassay have been under progressive development, refinement, and testing. In the past year, attention has focused on immunoradiometric assays, two-site immunoradiometric (labeled antibody) assays (sandwich assays), enzyme-mediated immunoassay techniques (EMIT), enzyme-linked immunosorbent assays (ELISA), and the labeled antibody membrane assay (LAMA). The chemical and mathematical equations describing these systems have been developed and used for computer simulation. Results provide a theoretical basis for the observed "paradoxical high dose hook effect" which occurs in many of these systems and suggests ways for improvement of these assay systems. The mathematical theory of optimization of RIA has been tested in practice and existing computer programs have been shown to be basically valid and useful guides to the experimentalist. Several new statistical methods for analysis of radioimmunoassay results have been developed.

PHS-6040
(12-75)

Objectives:

Development of mathematical and statistical methods for evaluation of radioimmunoassay systems and for optimization of radioligand assays.

Methods Employed:

Mathematical modeling, statistical analysis, computer simulation and practical laboratory radioimmunoassays for a variety of hormones, including hCG, estradiol and human growth hormone.

Major Findings:

The behavior of two-site immunoradiometric assays (sandwich assays) such as those used for serum hepatitis D virus, LH, FSH, growth hormone, insulin, etc., have been developed extensively. This makes it possible to predict the behavior of these rather complex nonequilibrium systems which are likely to involve a minimum of seven simultaneous reactions with 14 rate constants and a total of 19 parameters describing the system. A paradoxical high dose hook effect is shown to be the result of heterogeneity of the first antiserum (solid phase antiserum) and/or to incomplete washing of the first incubation. This analysis also applies to enzyme-linked immunosorbent assays (ELISA) and to labeled antibody membrane assays (LAMA). Statistical methods for analysis of results from two-site IRMA's have been developed and refined.

A mathematical theory of enzyme-linked immunoassay techniques (EMIT) have been developed. Again, this provides mathematical models for optimization of the several factors affecting performance of this assay, methods for statistical analysis of the resulting dose response curves have been developed, applied and tested.

Mathematical models for optimization of both equilibrium and nonequilibrium radioimmunoassays have been further refined and tested in application to RIA's for hCG and estradiol.

A quality control program for radioimmunoassays has been further developed, refined and streamlined. An exact definition of the minimal detectable dose of an assay has been developed. A new, simple method for testing of parallelism of dose response curves has been developed, which should be applicable to both linear and nonlinear dose response curves. Improved methods have been developed for the identification and characterization of nonuniformity of variance of bioassay and immunoassay dose response curves. A new bioassay program has been developed. New programs have been developed for fitting the mass action law equations (Scatchard plots), for fitting DNA-RNA and DNA-DNA hybridization experiments, and for a wide variety of related techniques.

Significance to Biomedical Research and the Program of the Institute:

This study has resulted in the development of a large number of computer programs for characterization of RIA, IRMA, ELISA, EMIT, LAMA, and a wide variety of other immunological techniques. These have been used by hundreds of investigators here at NIH and literally hundreds of copies of these programs have been distributed throughout the United States and overseas. These programs provide automation, considerable saving of professional and technician time, and most importantly provide optimal extraction of information from immunoassays, radioreceptor assays and bioassays. Further, they permit optimization of conditions for subsequent assays.

Proposed Course:

It is anticipated that further development and implementation of these studies will continue for the next several years.

Publications:

Faden, V. B. and Rodbard, D., Radioimmunoassay Data Processing, Third Edition, PB 246223, PB 246222, National Technical Information Service, Springfield, Va., 1975, 181 pp.

Rodbard, D., Faden, V. B., Knisley, S., and Hutt, D. M., RIA Data Processing: Logistic Method and Quality Control, PB 246224, National Technical Information Service, Springfield, Va., 1975, 100 pp.

Rodbard, D., Lenox, R. H., Wray, H. L., and Ramseth, D., Statistical Characterization of the Random Errors in the Radioimmunoassay Dose Response Variable, Clin. Chem., in press.

Faden, V. B., and Rodbard, D., Mathematics and Statistics of RIA: A Pictorial Survey, The Endocrine Society, RIA Course Syllabus, 1976, in press.

Rodbard, D., Comparison of Radioimmunoassay and Other Assay Methods, J. Clin. Endocrinol. Metab. 40: 895-897, 1975.

Rodbard, D. and Rodgers, R. C., The Kinetics of Radioligand Assays, in H. Antonaides (ed.), Hormones in Human Blood, Harvard University Press, 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00044-01 RR

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

In Vitro Studies on Primate Corpus Luteum Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Stouffer	Staff Fellow	RRB	NICHD
OTHER:	G. D. Hodgen	Chief, Section on Endocrinology	RRB	NICHD
	B. J. Gulyas	Senior Investigator	RRB	NICHD
	W. E. Nixon	Senior Investigator	RRB	NICHD
	D. K. Johnson	Chief, Vet. Med. & Surgery Sec.	VRB	DRS

COOPERATING UNITS (if any)

None

LAB/BRANCH

Reproduction Research Branch

SECTION

Section on Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 2.6	PROFESSIONAL: 2.0	OTHER: 6

SUMMARY OF WORK (200 words or less - underline keywords)

Isolated rhesus monkey luteal cells offer a model system for the in vitro study of primate corpus luteum function and assessment of the direct influence of various factors on the corpus luteum during its lifespan. Initial topics of interest in this project include: 1) Morphological and functional correlations between the intact corpus luteum and subsequent isolated luteal cells, 2) Comparison of the in vitro steroidogenic potential of luteal cells obtained from corpora lutea at various stages of the menstrual cycle and pregnancy, and 3) evaluation of the influence of potential luteotropic and luteolytic agents on in vitro steroidogenesis of macaque luteal cells.

PHS-6040
(12-75)

1. Project Description:

Objectives

The primary objective of this study is the development and utilization of an *in vitro* system of isolated luteal cells from the rhesus monkey to evaluate directly primate luteal function and the influence of potential luteotropic and luteolytic agents on the corpus luteum at various periods during its life-span.

Methods Employed

Luteal cells were dispersed from the corpus luteum of cycling and pregnant rhesus monkeys (*Macaca mulatta*) by collagenase digestion, followed by gentle mechanical disruption. Portions of the corpus luteum were also fixed for electron microscopic analysis of luteal ultrastructure. Blood samples were gathered from animals at luteectomy and serum progesterone concentration determined by radioimmunoassay. Dispersed luteal cells were incubated for up to 12 hours in Ham's F10 nutrient medium containing 25 mM HEPES buffer and graded doses of human chorionic gonadotropin (HCG), estradiol-17β (E_2) or HCG and E_2 combined. Progesterone production by macaque luteal cells *in vitro* was determined by radioimmunoassay of incubation medium samples. The radioimmunoassay developed in this laboratory was determined to be highly specific for progesterone and did not require solvent extraction of the incubation medium.

Major Findings

A procedure has been developed for dispersal of rhesus monkey corpus luteum into a population of predominately single, viable luteal cells.

The ability of luteal cells from cycling and pregnant rhesus monkeys to produce progesterone *in vitro* was a function of the stage of the menstrual cycle and gestation. Cells from mid-luteal phase of the menstrual cycle produced significantly greater amounts of progesterone under control conditions than cells from late luteal phase. Mid-luteal phase cells also responded to the luteotropic hormone, HCG, with enhanced P production; late-luteal phase cells were less sensitive to HCG. Luteal cells from early pregnancy secreted little progesterone *in vitro* and were unresponsive to HCG. However, as gestation continued, luteal cells progressively reacquired their steroidogenic capability and at term exhibited basal and HCG-stimulated progesterone production similar to cells from mid-luteal phase of the menstrual cycle.

Estradiol, which causes functional regression of the corpus luteum of primates *in vivo*, antagonized the steroidogenic effect of HCG in suspensions of macaque luteal cells *in vitro*. Higher E_2 doses also inhibited basal progesterone production by luteal cells.

Significance to Biomedical Research

Detailed information concerning the control of the functional lifespan of the primate, including human, corpus luteum is far from complete. The preparation of isolated rhesus monkey luteal cells developed in this project offers a model system for the *in vitro* study of primate luteal function and evaluation of the direct influence of various factors on luteal function. Knowledge of these factors may provide a mechanism for the manipulation of luteal function during the menstrual cycle and pregnancy in women, leading to a novel approach to fertility control.

Proposed Course

Additional studies will be performed to: 1) further examine the changes in luteal cell function during regression of the primate corpus luteum in the late luteal phase of the nonfertile menstrual cycle and during rejuvenation of the primate corpus luteum in advanced pregnancy, and 2) define the hormonal factors regulating luteal cell function during the menstrual cycle and pregnancy. Luteal cell function will be examined in the presence of potential luteotropic and luteolytic agents, such as the gonadotropic hormones, the estrogens and androgens, placental lactogen, prostaglandins, and others. These plans include the use of short term *in vitro* suspensions of dispersed luteal cells described previously and possibly long term cultures of luteal cells, if the appropriate facilities are available.

2. Publications:

Stouffer, R.L., Nixon, W.E., Gulyas, B.J., Johnson, D.K., and Hodgen, G.D.: *In Vitro* Evaluation of Corpus Luteum Function of Cycling and Pregnant Rhesus Monkeys: Progesterone Production by Dispersed Luteal Cells. *Steroids*, April 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00045-01 RR

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Ovarian Function in the Rhesus Monkey

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. L. Goodman	Staff Fellow	RRB	NICHD
OTHER:	G. D. Hodgen	Chief, Section on Endocrinology	RRB	NICHD
	W. E. Nixon	Senior Investigator	RRB	NICHD
	D. K. Johnson	Chief, Vet Med Surg Sec.	VRB	DRS

COOPERATING UNITS (if any)

None

LAB/BRANCH

Reproduction Research Branch

SECTION

Section on Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.8	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

To identify factors regulating initiation of follicle growth in primates the ovarian cycle of sexually mature rhesus monkeys was interrupted by surgical ablation of the preovulatory follicle and functioning corpus luteum. Monitoring of circulating levels of gonadotropins and ovarian steroids (estrogens and progesterone), as well as gross ovarian morphology indicates that the time and site of new follicle growth in the rhesus monkey are not random events; rather, they appear to be influenced locally by the dominant follicle or active corpus luteum. The objectives of the present study are aimed at interrelating the activities of the follicle and corpus luteum, along with pituitary gonadotropins, that contribute to the cyclic development of mature follicles in the primate. These studies can be related to problems of human female infertility (e.g. anovulation and short luteal phase) and pharmacologic control of fertility.

1. Project Description:

Objectives

Background and rationale. The development and use of sensitive radioimmunoassay techniques has provided a detailed description of the hormonal phenomenonology of the primate menstrual cycle. Whereas these kinds of studies have reinforced the notion of a key regulatory role of the ovary as an endocrine gland, the gametogenic activity of the ovary has received much less recent attention. It is now well established that the central events of the menstrual cycle--ovulation and corpus luteum formation--are triggered by a midcycle surge of gonadotropins. This abrupt acceleration in gonadotropin secretion is now known to be initiated by a surge of estradiol secreted by the maturing follicle itself. Thus, the ovary is also serving as a "Zeitgeber" in this biological rhythm to influence the temporal events of the menstrual cycle. Despite new methodologic sophistication, the regulation of follicular growth and development and corpus luteum function in primates remain poorly understood. An intriguing aspect of this problem is the occurrence of only a single ovulation each cycle, even though both ovaries are under a common influence of pituitary gonadotropins. This fact suggests some kind of between-ovary communication that remains to be elucidated.

Specific objectives:

1. To identify factors regulating the initiation of follicle growth in primates, using the ovarian cycle of the rhesus monkey as a model.

2. To study the nature of the selection whereby only one of the two ovaries develops the single dominant follicle.

3. To examine the possibility that the dominant follice and/or active corpus luteum has a local influence on the time and site of new follicle growth, since these appear not to be random events.

4. To test the hypothesis that ovarian steroids act on the ovary itself, along with gonadotropins, to contribute to the regulation of folliculogenesis and corpus luteum function.

Methods Employed

To identify factors regulating the initiation of follicle growth in primates, the ovarian cycle of sexually mature rhesus monkeys was interrupted by surgical ablation of the preovulatory follicle or functioning corpus luteum (CL). Patterns of circulating gonadotropins and ovarian steroids were monitored by radioimmunoassay of daily samples of femoral blood drawn before and after ablation localization of subsequent follicle growth or ovulation was determined at a second laparotomy 12-21 days later.

Major Findings

In 9 of 10 animals cautery of the largest visible follicle on Days 8-12 of the cycle abolished the midcycle LH surge; in the tenth animal the LH surge was found to have occurred on the day of follicle cautery. In 8 of these FSH and LH surges, preceded by or coincident with estradiol surges, occurred 10-14 days later. In 2 of these 8, corpus luteum function, as revealed by circulating progesterone levels, was temporally abbreviated (short luteal phase). No gonadotropin or estrogen surges were seen during the next 32 days of sampling in the other two, despite visible follicle growth. Unlike the first 8, both of these animals menstruated, beginning 4 or 9 days after cautery.

Removal of the CL on Days 15-17 (3-6 days after the midcycle LH surge) was folowed by menses about 4 days later in 6 of 6 animals. In 5 of these, gonadotropins remained at basal levels until 11-24 days after luteectomy when surges were observed. In the sixth, menses occurred again 25 days after luteectomy without an intervening LH surge. In both groups, new follicle growth was contralalateral to ablation in all except one animal.

In summary, our observations indicate that, by the middle of the follicular phase, the follicle destined to ovulate has been "selected," and that no other follicles were soon competent. This suggests that other follicles of that cohort could no longer be "rescued" to serve as a surrogate. The next follicle that developed or ovulated was apparently the product of a new "wave" of growth. Finally, our results suggest that the time and site of new follicle growth in the rhesus monkey are not random events; rather, they appear to be influenced locally by the dominant follicle or active CL.

Significance to Biomedical Research and the Program of the Institute

These studies clearly contribute to knowledge of the regulation of ovarian function in primates, especially the ovary's gametogenic activity. They can provide insights into the treatment of female infertility and programs of population control. The occurrence of short luteal phases (SLP) in 2 animals after follicle cautery indicates the potential of this procedure in studying SLP, known to be a cause of human infertility.

Proposed Course

To investigate the role of the corpus luteum in regulating the ovarian cycle, luteectomy will be combined with 2 kinds of replacement therapy of progesterone.

1. Silastic capsules as subcutaneous depots to maintain progesterone at luteal phase levels after luteectomy to determine if this hormone alone from the CL will affect follicle growth, since it is believed both estrogen and progesterone are secreted by the CL.

2. Intra-ovarian silastic pellets containing progesterone will be implanted at the site of luteecomy to ascertain if a direct ovarian influence is exerted by the CL.

To study the nature of the apparent communication between ovaries the procedure of hemiovariectomy at selected times of the cycle will be combined with the follicle cautery and luteectomy procedures above.

To establish an intraovarian effect of estrogen or progesterone, hypophysectomized animals bearing an intraovarian pellet of steroid in 1 ovary will be treated with exogenous gonadotropins to determine if the steroid containing ovary manifests a differential response from the contralateral gland.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00146-01 RR

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structure and Function of Chorionic Gonadotropins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. C. Chen	Senior Investigator	RRB	NICHD
OTHER:	G. T. Ross	Chief, Reproduction Research Branch	RRB	NICHD
	G. D. Hodgen	Chief, Section on Endocrinology	RRB	NICHD
	E. Gross	Chief, Section on Molecular Structure	RRB	NICHD
	S. Matsuura	Visiting Fellow	RRB	NICHD
	L. Lin	Staff Fellow	RRB	NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Reproduction Research Branch

SECTION

Section on Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 2.5	PROFESSIONAL: 2	OTHER: 0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Purpose of this project is to study chemical and biological properties of chorionic gonadotropins in tissues and biological fluids with particular emphasis on the isolation, purification and development of sensitive and specific detection methods to be applied in experimental and clinical endocrinology.

Project Description:

Objectives

1. To develop a radioimmunoassay highly specific for hCG and its application to laboratory diagnostic procedures.

2. To explore the mechanism of antigen-antibody interaction by means of systematic studies using synthetic peptides analogous to the antigen.

3. To apply the highly specific radioimmunoassay in search for hCG-like substances from normal, nonpregnant subjects.

Methods Employed

Radioimmunoassay, high speed digital computation, chromatographic methods (gel, ion exchange, partition and affinity), solid phase peptide synthesis.

Major Findings

An antiserum specific to hCG, but without affinity for hLH, was produced in a rabbit immunized with the bovine albumin conjugate of a tryptic peptide containing the carboxyl-terminal sequence unique to the hCG-β subunit. A radioimmunoassay system was developed which detects a minimum of 2-5 ng equivalents of highly purified hCG per tube. Examination of variety of gonadotropins and randomly collected 24 hour urine specimens in the form of kaolin acetone extracts revealed that the highly purified human gonadotropin preparations (hLH, hFSH, human urinary gonadotropins, pregnant mare serum gonadotropin), hTSH, hPL and kaolin-acetone extract containing marginal bioactivity and up to 800 mIU of immunoreactive gonadotropin thought not to contain hCG did not show crossreactivity. In contrast, activity was detected in human pituitary glycoprotein fraction, crude pituitary and urinary gonadotropin side fractions. Acetone precipitate of human monopausal women's urine was found to contain more crossreactive substance as compared with the equivalent kaolin-acetone extract. It appeared that the crossreactivity did not derive from either hLH or hFSH. More detailed studies are currently underway. This specific radioimmunoassay has been adapted with an established bioassay and two other radioimmunoassays for routine monitoring of patient urine extracts.

A series of peptides corresponding to the carboxyl-terminal segment of hCG-β subunit have been synthesized and their competitive binding properties in this radioimmunoassay system were examined. Based on binding affinity, the antibody recognition sites on the antigen reside on the last 15 amino acid residues of the carboxyl-terminal segment. Free carboxyl- and amino-termini are not essential for binding. However, deletion of eight amino acid residues from the carboxyl-terminal sequence abolished its immunoreactivity. In elucidation of antibody recognition sites, it appears that such a systematic approach, using synthetic peptide analogs, provides fundamental information concerning relationship between structural requirement and the nature of binding specificity. Peptides larger than pentadeca bind to antibody more strongly than hCG and; therefore, iodination of a high affinity synthetic peptide to be used as a tracer should improve sensitivity of the assay. On the other hand, low affinity peptides can be coupled to a solid support, improvising a favorable condition in purifying the specific antibody by affinity chromatography.

Since concentrated urine specimens from normal, nonpregnant subjects showed crossreactivity in this hCG specific radioimmunoassay, a gonadotropin fraction prepared from the similar source was subjected to gel chromatography. The crossreactive substance co-eluted with ^{125}I-labeled hCG marker as one completely overlapped peak which is distinctly separate from that of hLH. Two independent hCG radioimmunoassay systems with different characteristics also monitored the same peak.

Significance to Biomedical Research

These findings suggest that normal, nonpregnant subjects excrete hCG in urine

Proposed Course

1. To isolate and purify hCG-like substance(s) from urine and pituitary.

2. Physico-chemical characterization of the purified substance with aspects comparative with hCG and hLH.

3. To determine state and origin of its secretion.

4. To explore conditions and search for reagents capable of improving sensitivity of the hCG specific radioimmunoassay.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00147-01 RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Mechanisms of Action of Gonadotropins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	K. J. Catt, M.D., Ph.D., Head, Section on Hormonal Regulation
PI	M. L. Dufau, M.D., Senior Investigator, Reproduction Research Branch
Other	E. Podesta, M.D., Visiting Fellow, Reproduction Research Branch
	C. Williams, Ph.D., Staff Fellow, Reproduction Research Branch
	R. Rebar, M.D., Clinical Associate, Reproduction Research Branch
	R. Simpson, Ph.D., Visiting Fellow, Reproduction Research Branch
	S. Adamo, M.D., Visiting Fellow, Reproduction Research Branch

COOPERATING UNITS (if any)

LAB/BRANCH
Reproduction Research Branch

SECTION
Section on Hormonal Regulation

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.8	2.5	1.3

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to elucidate the biochemical pathways through which gonadotropic hormones control the metabolism and steroidogenic responses of the interstitital cells of the testis. This includes the characterization of gonadotropin receptors for LH and FSH in the testis, and the analysis of the coupling mechanisms between receptor occupancy and androgen synthesis. The two major questions examined are: (1) the role of cyclic AMP and protein kinase in the initiation of the steroidogenic and trophic actions of gonadotropin, and (2) the secondary sequence of events which leads to stimulation of the steroid biosynthetic pathway of the interstitial cell. This program has particular relevance to reproductive endocrinology, and is also aimed at a more general understanding of the mechanisms of action of peptide hormones upon target cells.

Objectives.

To define the mechanisms of action of luteinizing hormone (LH) upon steroidogenesis and growth in the interstitial cells of the testis.

Methods Employed.

Characterization of LH and FSH receptors by binding analysis in cell membranes and intact cells. Correlation of gonadotropin binding (using ^{125}I-hCG) with cyclic nucleotide production, protein kinase activation and testosterone synthesis in enzyme-dispersed interstitital cells from rat testis. Characterization of protein kinase activities in testis and identification of phosphorylated intermediates. Determination of RNA and protein synthesis during gonadotropin action. Measurement of activation of early biosynthetic steps in the sequence leading to testosterone formation.

Major Findings.

The use of collagenase-dispersed interstitial cells has provided a highly responsive preparation of target cells which are stimulated in vitro by physiologic concentrations of LH and chorionic gonadotropin (hCG). It is therefore possible to analyze hormone action at gonadotropin levels which are present in the circulation, rather than by exposure to pharmacological concentrations of hormone.

The role of RNA and protein synthesis in gonadotropin action was evaluated by precursor incorporation studies, and analysis of the effects of metabolic inhibitors upon gonadotropin-induced cyclic AMP and steroid synthesis. The actions of LH and hCG upon testosterone production were found to require continuous synthesis of RNA and protein by the target cells. However, gonadotropins did not stimulate RNA and protein synthesis while exerting their acute effect upon steroidogenesis, indicating that these biosynthetic processes are not directly involved in the mechanism of action of LH on testosterone production.

The isolated Leydig cells of the rat testis contain a limited number (about 6000/cell) of high affinity receptors for LH ($K_a = 2 \times 10^{10}$ M^{-1}). Occupancy of only 1% of the receptor population evokes a full steroidogenic response. The 'excess' 99% of the receptors are functionally active, in the sense of being coupled to adenylate cyclase, and occupancy leads to a progressive increase in cyclic AMP formation. However, the absence of a change in cyclic AMP in response to low concentrations of hormone which stimulate steroidogenesis (10^{-13}-10^{-12} M hCG) has suggested that the classical 'second messenger' role of cyclic AMP may not operate during the initiation of testosterone production in the testis.

To further investigate this possibility, the protein kinase of interstitial cells has been characterized and assayed during hormone stimulation in vitro.

The cyclic AMP-dependent protein kinase of rat interstitial cells was resolved into two forms by ion exchange chromatography, corresponding to the Type I and Type II holoenzymes observed in heart and adipose tissue. Enzyme activity was stimulated by cylic AMP over the range 0.25 to 8 μM, and the Km for ATP was 0.4 mM. The activity of the partially purified enzyme was stimulated 4-5 fold by cyclic AMP, and by incubation of dispersed cells with LH or hCG. Complete activation of protein kinase was produced in cells exposed to nanogram levels of hCG, but no change in kinase activity was observed during stimulation of steroidogenesis by low concentrations of hCG (below 10^{-11} M). Therefore, it is possible that a non-cyclic AMP pathway may operate during hormonal initiation of steroid secretion. Alternatively, a minor and compartmental change in cyclic AMP and protein kinase could operate as an intermediate step in gonadotropin action, and would require highly discriminating and sensitive procedures to be detectable. To approach this problem, more sensitive methods are being developed to detect endogenous activation of protein kinase by cyclic AMP, and to analyze the specific cellular proteins which are phosphorylated by protein kinase during hormone action.

Significance.

The hormonal control of Leydig cell function and androgen production is an essential component of male sexual development and reproductive capacity. In addition to its significance to the Reproduction Research program, this project has the potential to provide knowledge of the general actions of peptide and protein hormones upon target cells in tissues other than the gonads.

Proposed Course.

The role of cyclic nucleotides and protein kinase in gonadotropin action will be further analyzed by measurement of endogenous protein kinase activity and substrate phosphorylation in dispersed interstitial cells during LH stimulation in vitro. The role of the separate forms of protein kinase in gonadotropin stimulation of Leydig cells will be investigated, and translocation of catalytic kinase subunits to membrane and nuclear sites will be examined. Specific effects of gonadotropins upon RNA polymerase and messenger RNA synthesis during steroidogenesis will be sought, and the mechanism by which the biosynthetic conversion of cholesterol to pregnenolone is regulated by LH will be analyzed in the isolated interstitial cell preparation.

Publications.

Mendelson, C., Dufau, M. L., and Catt, K. J.: Gonadotropin Binding and Stimulation of Cyclic Adenosine 3'5'-Monophosphate and Testosterone Production in Isolated Leydig Cells. J. Biol. Chem. 240: 8818-8824, 1975.

Mendelson, C., Dufau, M. L., and Catt, K. J.: Dependence of Gonadotropin-Induced Steroidogenesis upon RNA and Protein Synthesis in the Interstitial Cells of the Rat Testis. Biochem. Biophys. Acta 411: 222-230, 1975.

Catt, K. J., and Dufau, M. L.: Basic Concepts of Mechanisms of Action of Peptide Hormones. Biology of Reproduction 14: 1-15, 1976.

Podesta, E. J., Dufau, M. L., and Catt, K. J.: Characterization of Two forms of Cyclic 3'5'-Adenosine Monophosphate-Dependent Protein Kinase in Rat Testicular Interstitial Cells. Molecular and Cellular Endocrinology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00148-01 RR

PERIOD COVERED

July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Ontogeny of Gonadotropin Receptors and Leydig Cell Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	K. J. Catt, M.D., Ph.D., Head, Section on Hormonal Regulation, RRB
PI	M. L. Dufau, M.D., Senior Investigator, RRB
Other	J-M. Ketelslegers, M.D., Visiting Fellow, RRB
	A. Hsueh, Ph.D., Visiting Fellow, RRB

COOPERATING UNITS (if any)

Department of Medicine, University of Texas Southwestern Medical School, Dallas, Texas

LAB/BRANCH

Reproduction Research Branch

SECTION

Section on Hormonal Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to clarify the developmental patterns of gonadotropin receptors and their regulatory mechanisms during fetal development and sexual maturation. Developmental studies include the ontogeny of LH receptors in the fetal rabbit testis and ovary, and analysis of the postnatal changes in LH and FSH receptors during maturation of the rat testis. In addition, the role of FSH in the control of Leydig cell function and LH responsiveness is being studied during normal sexual development, and in the testis of the immature hypophysectomized rat.

Objectives.

To obtain detailed information about the developmental aspects and regulation of gonadotropin receptors during fetal life and sexual maturation.

Methods.

The concentration and affinity of specific receptor sites for LH and FSH were determined in testis and ovary by quantitative binding studies with radioiodinated hLH, hCG and hFSH. All measurements were corrected for the biologically determined specific activity of the labeled hormone, and also for the maximum binding activity of the tracer preparation. For measurement of receptor concentration alone, without determination of binding affinity, assay of binding by serial aliquots of gonadal homogenates in the presence of excess labeled hormone have been shown to provide data similar to that derived by the more complex saturation procedure with fixed binding site concentration and increasing concentrations of labeled hormone.

Studies on the ontogeny of gonadotropin receptors were performed in the fetal rabbit testis. The development of receptors during sexual maturation was analyzed in the male rat. The actions of FSH upon testicular function were studied in the hypophysectomized immature male rat.

Major Findings.

(1) LH Receptors During Differentiation of Testis in Rabbit Embryo. These studies, performed in collaboration with Drs. Jean Wilson and Patrick Walsh, showed that the concentrations of LH receptors and testosterone in the fetal testis were very low on days 17 and 18, and increased strikingly by day 19. More detailed analysis of the time course of receptor development showed that both receptor content and testosterone content increased suddenly over a 12-hour period on day 18. This time sequence for development of LH receptors and testosterone synthesis was exactly correlated with the histological appearance of the endoplasmic reticulum characteristic of the differentiated Leydig cell. The binding affinity of the fetal testis receptors for LH was identical with that of the adult testis receptors (3×10^{10} M^{-1}) from their earliest time of development at 18.5 days of gestation. In the fetal ovary, LH receptors and testosterone were low or undetectable at all stages of gestation. These studies have shown that the Leydig cells differentiate rapidly within a few hours on day 18, and acquire a full complement of LH receptors and enzyme pathways necessary for testosterone synthesis. These observations are compatible with the possibility that Leydig cell development and the onset of testosterone synthesis are under gonadotropic control in the fetus.

(2) Developmental Changes in Testicular Gonadotropic Receptors During Sexual Maturation. The concentration of LH and FSH receptors in the testis showed marked changes during sexual maturation, and the affinity of LH receptors was about ten-fold greater than that of FSH receptors throughout development. FSH receptor concentration peaked at 10-15 days of life, then remained constant through adult life. Plasma FSH levels rose rapidly from days 15 through 38, accompanied by a major rise in LH receptor concentration in the

testis. Plasma testosterone levels then rose markedly between days 35 and 55. These changes occurred in the absence of a rise in plasma LH, suggesting that testis maturation is initiated by increasing concentrations of plasma FSH, with induction of LH receptors and an increasing testosterone response to relatively constant levels of plasma LH.

(3) Actions of FSH upon LH Receptors and Steroidogenic Responsiveness in the Immature Rat Testis. FSH has been previously demonstrated (Swerdloff and Odell) to influence the in vivo responsiveness of the hypophysectomized rat testis to administered LH. To further investigate the mode of action of FSH in the restoration of endocrine function, measurements of gonadotropin receptors and in vitro Leydig cell responses to LH were performed upon testes from FSH-treated hypophysectomized rats. These studies showed that the enhanced responsiveness of the testis from FSH-treated rats is partly attributable to increased LH receptors in the Leydig cells. This effect upon LH receptors in the interstitial compartment of the testis could be due to indirect or direct actions of FSH upon Leydig cell function. A mechanism for such a direct action of FSH was revealed by the demonstration of a small number of FSH receptors in the immature Leydig cell. Such receptors are not present in the Leydig cells of the adult animal.

Thus, FSH treatment causes an increase in testis weight and LH receptor concentration, and enhanced sensitivity of the steroidogenic response to LH. The actions of FSH on LH receptors and steroidogenic responsiveness appear to be mediated through binding to FSH receptors present in the interstitial cells of the immature rat testis.

Significance.

The analysis of gonadotropin receptors during fetal life and sexual maturation will provide valuable information about the mechanisms by which male phenotypic development is determined, and about the control of gonadal function during puberty. These topics are closely related to the program of the Reproduction Research Branch, since they deal with fundamental aspects of sexual development in the male.

Proposed Course.

Studies on the ontogeny of the LH receptor will be extended in the fetal rabbit during contined collaboration with Dr. Jean Wilson. The development of androgen biosynthetic capacity and LH receptors will be examined during culture of gonadal explants, and in vitro responses to gonadotropin will be sought with cultured gonads. The role of gonadotropin in the control of fetal Leydig cell function in vivo will also be studied, and the levels of gonadotropin in the fetal pituitary and circulation will be measured.

Further studies on the changes in receptors during maturation will be performed in the immature male rat, to determine the precise role of FSH and gonadal steroids in the induction of the steroidogenic response of the testis to LH. The relationships between gonadotrophic hormones and steroids during development will also be examined by determination of estrogen and androgen receptors in the maturing rat testis.

Publications.

Catt, K. J., Dufau, M. L., Neaves, W. B., Walsh, P. C., and Wilson, J. D.: LH-hCG receptors and testosterone content during differentiation of the testis in the rabbit embryo. Endocrinology 97: 1157-1165, 1975.

Catt, K. J., Podesta, E. P., Ketelslegers, J.-M., and Dufau, M. L.: Gonadotropin receptors and regulation of interstitial cell function in the testis. Proc. Soc. Dev. Biol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00149-01 RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Bioassay of Serum Luteinizing Hormone (LH) and Chorionic Gonadotropin (CG)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	M. L. Dufau, Senior Investigator, RRB, NICHD
PI	K. J. Catt, Chief, Section on Horm. Reg., RRB, NICHD
Other	G. Hodgen, Chief, Endocrinology Section, RRB, NICHD
	R. J. Sherins, Senior Investigator, RRB, NICHD
	R. Rebar, Clinical Associate, RRB, NICHD

COOPERATING UNITS (if any)
Dept. of Gyn., Harvard Med. School, Mass. General Hospital, Boston, Mass.
Dept. of Physiology, Medical College of Virginia, Richmond, Virginia

LAB/BRANCH
Reproduction Research Branch

SECTION
Section on Hormonal Regulation

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	0.25	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

A comprehensive study on the bioactivity of luteinizing hormone and chorionic gonadotropin in man and other species has been performed utilizing a newly developed and highly sensitive in vitro bioassay for serum LH and CG.

PHS-6040
(12-75)

Objectives.

1) Development and optimization of in vitro bioassay for serum LH in the human. 2) Application of the newly developed bioassay to measurement of LH in human serum in physiological and pathological states, and during stimulation with Luteinizing Hormone Releasing Hormone (LHRH). 3) Comparative studies with radioimmunoassay and analysis of bio:immuno (B:I) ratios in normal subjects and in pathological states. 4) Measurement of hCG by in vitro bioassay in induced and spontaneous pregnancy at early stages to determine the earliest time at which biologically active hCG can be detected. Comparative studies with radioligand-receptor assays and specific β-subunit radioimmunoassay. 5) Follow-up of patients with choriocarcinoma on oral contraceptive therapy during remission, relapse and treatment periods. 6) Development and optimization of in vitro bioassay for serum LH in the rhesus monkey, and application of the bioassay to physiological states and durin stimulation with LHRH.

Methods Employed.

Newly developed in vitro bioassay for serum gonadotropin. Radioimmunoassay (RIA) for LH and β-subunit of hCG.

Rat Interstitital Cell Testosterone (RICT) Assay for LH and hCG. A highly sensitive bioassay for LH and hCG has been developed by utilizing the testosterone response of collagenase-dispersed rat Leydig cells to gonadotropic stimulation in vitro. Testosterone production by dispersed interstitial cells is stimulated by human, ovine, bovine, porcine, rat, rabbit and monkey LH and by hCG, monkey CG and PMSG. The RICT assay gives parallel dose-response curves for all steroidogenic gonadotropins tested, and thus permits cross-species comparison of the intrinsic biological activities of native and modified gonadotropins. The sensitivity of the RICT assay is equal to or higher than that of radioimmunoassay, and permits measurement of picogram quantities of gonadotropin

Bioassay of Human LH in Serum. Optimum conditions were developed for the bioassay of serum gonadotropins during incubation with dispersed Leydig cells. The within-assay and between-assay reproducibility at normal plasma LH levels was ±10% (CV) and ±15%, respectively. The precision of the assay was relatively high, with λ of 0.04 and Finney's 'g' value of 0.004. This method permits bioassay of circulating LH in normal subjects, in serum samples of 200-100 μl. The higher gonadotropin levels in postmenopausal, agonadal and LHRH-stimulated samples can be measured in 1-10 μl serum. The method has been applied to measurement of serum LH in males, normal premenopausal females throughout the menstrual cycle, and postmenopausal females. The serum LH values obtained by RICT assay in human subjects have been generally higher than those measured by RIA, although the two values were similar in normal premenopausal females.

Analysis of Bio:Immuno Ratio (B:I). The B:I potency ratio in cycling females was usually close to unity, although individual women sometimes showed ratios of 1.5-2.0. No consistent change in B:I ratio was observed during the menstrual cycle, and the bioactive serum LH profiles were always identical with those derived by radioimmunoassay. This finding confirms the validity of immunoreactive LH values as an index of biologically active LH present in the circulation. In males and postmenopausal subjects, the B:I ratio was consistently elevated, with bioassay values 2.5 times higher than those measured in the same samples by RIA. The explanation for the relatively higher B:I ratio in males and postmenopausal females is not readily apparent. Several explanations could be entertained, such as the gonadal status of the individual, or the rate of secretion of gonadotropin from the pituitary. In these studies, the higher B:I ratios were observed in subjects with relatively low estrogen secretion and/or increased LH secretion rate, i.e. men, postmenopausal females.

Biologically Active LH Levels in Serum of Normal Subjects Following Administration of LHRH. The effects of LHRH stimulation upon plasma LH levels were investigated at various stages of the menstrual cycle, and in normal males and postmenopausal females. The stimulation studies confirmed the B:I ratios of earlier studies, and provided additional evidence for the biological validity of LH levels measured in serum by in vitro bioassay. In the normal female, the elevations of bioactive LH following LHRH were generally accompanied by a significant rise in B:I ratio except during the early follicular phase, when the responses were small and the ratio did not change from unity. No significant changes in B:I ratio from control values were observed during LHRH stimulation of serum LH levels in normal males and postmenopausal females.

These studies suggest that circulating LH molecules exhibit a relatively higher biological activity when the rate of biosynthesis and release of gonadotropins is increased. It is also conceivable that the serum LH with higher B:I ratio, released during LHRH stimulation in the late follicular and luteal phases of the menstrual cycle, represents secretion from a further pool of pituitary LH.

Serum LH Bioactivity in Endocrine and Gonadal Disease. Studies were performed in agonadal males, and patients with Klinefelter's Syndrome, germinal aplasia (spontaneous and following chemotherapy) and hypopituitarism. Elevated levels of bioactive LH were observed in some patients with Klinefelter's Syndrome and germinal aplasia when only small increases were shown by RIA. This finding suggests that the in vitro bioassay will be of value to assess LH secretion in gonadal disorders.

In female patients, studies were performed in Turner's Syndrome and in women with choriocarcinoma. In Turner's Syndrome, the B:I ratio was markedly increased, to the level observed in postmenopausal women. The possible role of estrogen in modifying the B:I ratio will be further investigated in these conditions. In patients with choriocarcinoma, bioactive hCG levels were higher than those measured by RIA, and the in vitro bioassay could detect hCG in the serum during chemotherapy when circulating hCG was no longer detectable by RIA.

Bioassay of Serum Levels in the Rhesus Monkey. Biologically active LH concentrations were readily measured in the Rhesus monkey by the in vitro bioassay. The sensitivity of the bioassay is higher than that of RIA, and the assay does not recognize the spurious LH activity detected in monkey serum by certain RIA methods. The bioactive serum LH levels in the female Rhesus monkey are significantly higher than in women, and show appropriate physiological fluctuations and responses to administration of gonadal steroids.

Significance to Biomedical Research.

The in vitro bioassay for LH has provided, for the first time, a reliable method for measurement of LH in human serum. The ability to measure the biological activity of circulating LH in gonadal and pituitary disease will permit a more accurate evaluation of LH secretion in these conditions. Elucidation of the factors influencing B:I ratio could provide information about the modulation of qualitative changes in LH, as well as the previously measured quantitative changes during endocrine disease. The high sensitivity of the assay for LH and hCG has potential value for studies on treatment of choriocarcinoma, and for monitoring the effects of contraceptive agents on circulating LH in primate models.

Proposed Course.

1. Further investigations to gain insight into the nature of the B:I differences observed between the different groups of subjects will include: a) examination of different LH pools released from the pituitary during stimulation studies, b) physical characterization of serum LH of the different groups of subjects, c) examination of different groups of patients, such as Turner's Syndrome and postmenopausal women, during acute and chronic estrogen treatment.

2. Evaluation of more patients with germinal aplasia to determine the value of the bioassay to evaluate Leydig cell function.

3. Continuation of the evaluation of choriocarcinoma patients.

4. Application of the bioassay to rat LH, to study biosynthesis of LH in the pituitary.

Publications.

Dufau, M. L., and Catt, K. J., in Methods in Enzymology XXXIX, Part D, Hardman, J. and O'Malley, B. W. (eds.), Academic Press, New York, 1975, pp. 252.

Dufau, M. L., Pock, R., Neubauer, A., and Catt, K. J., In Vitro Bioassay of LH in Human Serum: The Rat Interstitial Cell-Testosterone (RICT) Assay, J. Clin. Endocrinol. Metab., in press.

Dufau, M. L., Beitins, I., McArthur, and Catt, K. J. Qualitative Differences in Serum LH, in Third NICHD Testis Workshop, Raven Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00150-01-RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Characterization and Purification of LH/hCG Receptors and Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	M. L. Dufau, M.D., Senior Investigator, RRB
PI	K. J. Catt, M.D., Ph.D., Head, Section on Hormonal Regulation, RRB
Other	M. Conti, M.D., Visiting Fellow, RRB
	J. Harwood, M.D., Staff Fellow, Section on Hormonal Regulation, RRB

COOPERATING UNITS (if any)

LAB/BRANCH
Reproduction Research Branch

SECTION
Section on Hormonal Regulation

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	1.4	1.3

SUMMARY OF WORK (200 words or less - underline keywords)

This is a long range project on the solubilization, characterization and purification of LH/hCG and FSH receptors and adenylate cyclase of testis and ovary. In addition to analyzing the properties of gonadotropin receptors and adenylate cyclase, these studies will lead ultimately to an analysis of the physical and functional relationships of the binding site and the enzyme complex in the cell membrane and in solution.

Objectives.

1) Solubilization of LH/hCG and FSH receptors from gonadal target cells. 2) Binding analysis of the solubilized preparations, comparative studies with particulate receptors. 3) Characterization of gonadotropin receptors. 4) Large scale purification of LH/hCG receptors for structural analysis and antibody formation. 5) Characterization of particulate testicular and ovarian adenylate cyclase. 6) Solubilization of cyclase from testicular and ovarian membranes. 7) Relationship between receptor site and adenylate cyclase enzymatic complex.

Methodology.

Assay of particulate and soluble receptors is employed for the separation of receptor-hormone complex and free hormone by millipore filtration and polyethylene glycol precipitation; adenylate cyclase assay; cyclic AMP radioimmunoassay; sucrose and cesium chloride gradients; ultracentrifugation analysis; gel filtration and affinity chromatography; SDS gel electrophoresis.

Solubilization of LH/hCG Receptors. The particulate high affinity gonadotropin receptors of rat testis and ovary are readily solubilized by treatment with nonionic detergents, such as Triton X-100 and Lubrol. Extraction with Triton X-100 has been found to yield soluble receptors with similar affinity to the parent preparation (Ka = 10^{10} M^{-1}) and specificity for CG and LH. Such preparations have been utilized for determination of physical characteristics of the soluble receptor site. The rate of gonadotropin binding was found to be temperature-dependent. The second-order association rate constant calculated from the binding velocity and binding capacity of the soluble testis receptor is 6.1×10^5 M^{-1} min^{-1} at 4°C. Dissociation of ^{125}I-labeled hCG from soluble receptor-hormone complex was found to occur extremely slowly, with a first order dissociation rate constant of 1.2×10^{-4} min^{-1}. The latter property was of great advantage for subsequent characterization of soluble preparations and for purification studies.

Physical Characteristics of Solubilized Testicular Receptors. After extraction of receptors from membrane rich preparations with various nonionic detergents, fractionation of free receptors, equilibrium mixtures of hormone-receptor complex and free ^{125}I-hCG preparations was performed by gel filtration and ultracentrifugation. The apparent molecular weight of the free receptor was 194,000, and that of the hormone-receptor complex 224,000. The difference between both forms is consistent with binding of one molecule of gonadotropin by each molecule of the receptor. The Stokes radii of the Triton-solubilized receptors are close to 6.4 nm, and the sedimentation constants in sucrose gradients are 6.5S for free receptors, 7.5S for the hCG-receptor complex formed after extraction and 8.8S for the complex extracted from gonadal particles prelabeled with ^{125}I-hCG.

Solubilized Ovarian Gonadotropin Receptors. The physical characteristics of the soluble ovarian receptors have been found to be almost identical with those of the testis receptors. In each case, the receptor behaves as an asymmetric molecule and appears to contain phosholipid moieties in addition to the major binding protein. Lubrol extraction of the free receptor appears to yield the 200,000 molecular species and a smaller component with binding activity of approximately 60,000 MW. The ovarian receptors solubilized after labeling in vivo by administration of radioiodinated gonadotropins to PMSG-hCG treated rats showed identical physical properties to the soluble receptors extracted by detergent treatment of particulate ovarian receptors labeled with ^{125}I-hCG in vitro.

Characterization of a Soluble Cytosolic Gonadotropin Receptor for LH/hCG in Luteinized Rat Ovaries. Soluble receptors were also observed in the supernatant solution following homogenization of luteinizing rat ovaries to prepare particulate receptor sites. The naturally soluble receptor sites present in the 360,000 g supernatant fraction were associated with floating lipid fractions, and could not be detected in the clear ovarian supernatant or in similar fractions prepared from interstitial cells or homogenates of the normal rat testis. Such receptors appeared on the first day of PMS treatment, representing 10% of the total receptor population on the second day, and declining to 2-3% after 8 to 9 days of treatment. The physicochemical properties of the soluble ovarian receptors were similar to those derived for detergent-soluble extracted particulate ovarian receptors in the soluble phase. The affinity constant of the naturally soluble receptor sites for ^{125}I-hCG was 0.73×10^{10} M^{-1} and that of the receptors solubilized by Triton X-100 was 0.72×10^{10} M^{-1}. The sedimentation pattern of the cytosol receptors during sucrose gradient centrifugation showed extensive aggregation which converted in Triton X-100 to the 6.5S component previously characterized in detergent extracts of particulate receptors from the rat ovary and testis. The presence of a spontaneously soluble LH/hCG receptor in ovarian cytosol fractions suggests that rapid synthesis and assembly of receptors in ovaries of PMSG-hCG treated rats is accompanied by increased production of cytosolic receptor precursors.

Purification of LH/hCG Receptors from Testis. Specific gonadotropin receptors of the rat testis were purified 15,000-fold after detergent extraction by affinity chromatography on agarose coupled to human chorionic gonadotropin. Receptors were eluted in the free form at pH 3.2 and did not aggregate in the absence of detergent. The purified receptors displayed about 50% of the maximum theoretical binding capacity, and retained the hormonal specificity of the original gonadotropin receptors of the cell membrane. The most highly purified preparation was demonstrable by SDS gel electrophoresis as a single component with approximate molecular weight of 90,000. Thus, the original detergent-extracted receptor of 194,000 could consist of a dimer composed of subunits of 90,000 molecular weight. Ovarian membrane and cytosol LH/hCG receptors can also be purified by the above procedure. However, these lipid-rich preparations give recovery of 30-40%, compared to nearly 100% for the testicular receptors, during affinity chromatography, and 2,000-fold purification versus 15,000-fold for the testicular gonadotropin receptor. These results favored the use of the testicular receptors for large-scale purification and quantities of the order of 20 μmg/ml are produced from approximately 1000 adult rat testes. We have commenced attempts to obtain antibodies to the receptor in these preparations.

Solubilization of FSH Receptors. Soluble FSH receptors have been obtained by Triton-extraction of testis homogenates prelabeled with ^{125}I-FSH. The efficacy of extraction was 20-40% and other non-ionic detergents, such as Lubrol PX or WX, were less effective solvents. The properties of testicular FSH receptors have been determined by gel filtration. In contrast to the solubilized LH receptors, considerable dissociation of the hormone from receptor sites was observed during fractionation procedures. The Kav of the hormone receptor complex was 0.30, corresponding to a Stokes radius of 65 A, similar to the soluble LH receptor complex found in vitro and in vivo. The Ka of the soluble receptor is 6.2×10^9 M^{-1}, somewhat higher than the association constant for the particulate FSH receptor of Ka 2.6×10^9 M^{-1}.

Testicular and Ovarian Adenylate Cyclase. The marked increase of cAMP production stimulated by higher degrees of receptor occupancy in the intact Leydig cell indicated that a high proportion of testicular LH receptors are functionally related to adenylate cyclase in the plasma membrane. Studies on the activation of adenylate cyclase in the particulate testis preparations, either the 600 g fraction of disrupted interstitial cells or membranes fractionated in discontinuous sucrose gradients, showed increases of enzyme activity of 8-10 fold over basal levels in the presence of 10 mM fluoride. The stimulation of adenylate cyclase produced by hCG or ovine LH was usually quite small and non-linearity of the enzyme reaction was observed in the unfractionated preparations after 5 minutes incubation. However, the reaction was linear up to 30 min. in the membrane-rich fraction of the sucrose gradients. The small degree of stimulation of the enzyme activity by the trophic hormone is an unexpected finding since the in vitro cell preparations are sensitive to 10^{-13} to 10^{-12} M hCG for steroidogenic responses and 10^{-12} to 10^{-10} M for cAMP responses. The presence of an inhibitor of the enzyme reaction and the role of adenosine as well as substrate availability is under study. In these studies there was no evidence of a nucleotide requirement. In contrast, the enzyme in ovarian homogenates of PMS/hCG-treated rats was stimulated 2-fold by LH. In the presence of Gpp(NH)p, both basal and LH, but not fluoride activity were stimulated, indicating the presence of a nucleotide requirement for LH action in the ovary.

Detergent-Soluble Adenylate Cyclase. The relationships between hormone binding sites and adenylate cyclase were studied in detergent extracts of particulate testis and ovarian receptors. Both basal and fluoride-stimulated activities of the particulate enzyme were markedly increased in the presence of detergents, and 50% of the enzyme activity was recovered in the 360,000 g supernatant. The highest recovery and activation (10-fold) of adenylate cyclase was obtained with 0.5% Lubrol PX. The particulate and soluble enzymes were more active in the presence of Mn^{++}, and the detergent-extracted ovarian cyclase showed a small response to gonadotropin. Fractionation of Lubrol-solubilized testis and ovarian particles on Sepharose 6B showed two peaks of free gonadotropin receptors. The binding peak of Kav 0.32 corresponded to the sites previously extracted from gonads, and was coincident with the elution profile of basal and fluoride-stimulated cyclase activity. The second peak with Kav 0.56 was not accompanied by a detectable change in cyclase activity. This suggests that the receptor-cyclase complex is eluted as the peak of larger molecular size, while the cyclase-free second peak could represent the dissociated binding sites.

Soluble Adenylate Cyclase of Testis Cytosol. In studies analyzing the distribution of adenylate cyclase activity during differential centrifugation of fragmented interstitial cells, a spontaneously soluble adenylate cyclase was found in the 300,000 g supernatant. This enzyme requires Mn^{++} for activity, has a Km of 1.5 mM for ATP and is not activable by fluoride or trophic hormone. The soluble adenylate cyclase is extremely stable and can be kept at 4° to 16° C without loss of activity for at least one month. The enzyme does not aggregate during gel filtration in Sepharose 6B, and the enzyme activity was eluted with a Kav of 0.62 corresponding to a radius of 29 Å. On sucrose gradient sedimentation the S value was 3.4. There was a quantitative recovery of enzyme activity during fractionation and treatment with detergents did not change sedimentation patterns or elution profiles from column chromatography. The enzyme behaves as a globular protein and has an approximate molecular weight of 40,000. The significance of this soluble cyclase in the trophic hormone stimulatory response will be a topic of future investigations. The stability of the enzyme and the abundant activity observed in this preparation offer the potential for isolation of the soluble adenylate cyclase and study of its properties in the purified state.

Proposed Course.

1) Large-scale purification of LH/hCG recpetors for antibody formation and physico-chemical characterization.

2) To proceed with studies on the cytosolic LH/hCG receptors and their role as precursors of membrane receptors.

3) Purification of soluble adenylate cyclase and analysis of its role in hormone action and/or cell metabolic functions.

Significance to Biomedical Research.

The availability of purified receptors will permit a more detailed study to be performed on the nature and control of binding proteins and of the signal by which the occupation of receptors is conveyed to the cell interior. In addition, blockade of receptors by antibodies to the purified binding sites or by modified gonadotropins with inhibitory activity will be of value during *in vivo* and *in vitro* studies on receptor function. It can also be expected that greater knowledge of the basic mechanism of gonadotropin action will provide new possibilities for modulation of the biochemical processes which are responsible for the maintenance of fertility.

Publications.

Dufau, M. L., Ryan, D. W., Baukal, A., and Catt. K. J.: Gonadotropin Receptors: Solubilization and Purification by Affinity Chromatography. *J. Biol. Chem.* 250: 4822, 1975.

Dufau, M. L., Podesta, E. J., and Catt, K. J.: Physical Characteristics of the Gonadotropin Receptor Hormone Complex Formed In Vivo and In Vitro. *Proc. Nat. Acad. Sci.* 72: 1727, 1975.

Dufau, M. L., Baukal, A. J., Ryan, D., and Catt, K. J.: Properties of Detergent Solubilized Adenylate Cyclase and Gonadotropin Receptors of Testis and Ovary. J. Mol. Cell. Endocrinol., in press.

Catt, K. J., Conti, M., and Dufau, M. L.: Isolation and Properties of Ovarian Gonadotropin Receptors. In The Endocrine Function of the Human Ovary (V.H.T. James and M. Serio, eds.) Academic Press, New York, in press.

Catt, K. J., and Dufau, M. L.: Gonadotropin Receptors of Testis and Ovary. In Hormone-Receptor Interactions: Molecular Aspects (G. J. Levey, ed.), Raven Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00151-01 RR

PERIOD COVERED

July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Gonadotropin Receptors in Target Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

LAB/BRANCH

Reproduction Research Branch

SECTION

Section on Hormonal Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

SUMMARY OF WORK (200 words or less - underline keywords)

This project is designed to provide information about the regulation and biosynthesis of gonadotropin receptors in the adult testis and ovary. The dependence of gonadotropin receptors upon pituitary hormones and gonadal steroids has been examined in the adult rat. Also, the biosynthesis of LH receptors has been studied in the luteinized ovaries of rats treated with gonadotropins.

PHS-6040
(12-75)

Objectives.

To clarify the mechanisms which regulate the biosynthesis, expression and turnover of gonadotropin receptors in their respective gonadal target cells.

Methods Employed.

Gonadotropin receptors were measured by quantitative binding assays with ^{125}I-labeled LH and hCG. Incorporation of labeled precursors into cell protein and gonadotropin receptors of luteinized rat ovaries was examined by conventional methods and by affinity chromatography. The dependence of testis receptors on pituitary and gonadal hormones was studied in hypophysectomized rats. A technique for detecting LH receptors and receptor occupancy by immunofluorescence was developed and applied to the testes of normal and hypophysectomized rats.

Major Findings.

(1) Pituitary Control of Gonadotropin Receptors. Hypophysectomy of male rats was followed by a rapid fall in LH receptors, to about 40% of the control value over a period of 3 days. However, FSH receptors were relatively uninfluenced by hypophysectomy and did not show a significant decline until about three weeks after surgery. These findings indicate that a proportion of the LH receptor population is pituitary-dependent, and that certain LH receptors and most FSH receptors are relatively independent of pituitary support. The fall in LH receptors measured in testis homogenates was also demonstrated in Leydig cell dispersions from testes of hypophysectomized rats, and was accompanied by a marked fall in steroidogenic responsiveness to LH of whole testis and dispersed Leydig cells in vitro. In contrast, the cyclic AMP response of the hypophysectomized rat testis to LH was significantly enhanced above the control value. Treatment of hypophysectomized rats with various combinations of pituitary hormones (LH, FSH, GH, Prolactin) did not restore the LH receptor content of the testis to control levels in the adult rat.

These studies have demonstrated that hypophysectomy has complex effects upon Leydig cell function, with partial receptor loss, marked decline of steroid responsiveness, and significant enhancement of cyclic AMP production. The dissociation between receptors and cyclic AMP formation is unexpected, but could result from altered phosphodiesterase activity after hypophysectomy. Also, we have previously shown that only 1% of the LH receptors need be occupied by hormone to evoke a full steroid response, whereas the majority of the receptors are functionally coupled to adenylate cyclase. Assuming that all receptors are equally effective in eliciting a steroid response, the marked decrease in testosterone production during incubation with LH seems unlikely to be directly attributable to the observed effects of hypophysectomy upon LH receptor content in the testis. It is more likely that the decay of labile gonadotropin-dependent components in the steroid biosynthetic pathway is responsible for the loss of the testosterone response, particularly since the cyclic AMP response is retained and enhanced.

(2) Immunofluorescence Labeling of Gonadotropin Receptors. Specific receptor sites for LH (and hCG) in the rat testis were detected by an indirect immunofluorescence method, employing rabbit antiserum to hCG and fluorescein-conjugated goat antiserum to rabbit gamma globulin. This procedure permitted gonadotropin receptors in dispersed interstitial cells to be demonstrated during occupancy by endogenous LH (which cross-reacted with the anti-hCG serum) or after exposure to exogenous gonadotropin (hCG) to saturate the receptor sites.

By this method, about 40% of the interstitial cells showed marked fluorescence after exposure to hCG. After hypophysectomy, the proportion of fluorescent cells decreased by about 50% at 6 days, and remained at this level for 3 weeks. Such a partial loss of LH receptors is consistent with the observations cited above, based upon binding of ^{125}I-labeled hormone following hypophysectomy. Since the fluorescence method is not quantitative on a "per cell" basis, it demonstrates that loss of receptors occurs in a proportion of the interstitial cell population, rather than as a more uniform loss from all interstititial cells. These studies also demonstrated that occupancy of receptors by endogenous hormone decreased in a serial fashion after hypophysectomy, but revealed that occupancy did not become undetectable until almost two weeks had elapsed.

Such prolonged occupancy of the receptors by endogenous hormone suggests that at least a proportion of the receptor-hormone complexes dissociate slowly in vivo, and that such occupied receptors could be rendered inaccessible to further hormone stimulation for a prolonged period. The observation also raises the possibility that occupied receptors could be inactivated or degraded, rather than reutilized during further hormonal stimulation by gonadotropin. The use of immunofluorescent methods to detect peptide hormone receptors has considerable potential for investigation of the turnover and regulation of receptors within the plasma membrane of target cells.

(3) Synthesis of LH Receptors in the Luteinized Ovary. To investigate the biosynthesis of receptors, the luteinized ovaries obtained by PMSG and hCG treatment of immature rats have been employed for in vitro binding and incorporation studies. The marked ovarian hyperplasia and luteinization which follows PMSG/hCG treatment is accompanied by a progressive and marked increase in LH receptors, to about 300-fold above the original ovarian content. During the gonadotropin-induced rise in LH receptors, the experimental animals were given large quantities of tritiated aminoacids parenterally. Subsequent isolation of membrane-rich fractions from ovarian homogenates showed substantial incorporation of radioactivity, but no label was present in LH receptors isolated by detergent solubilization and affinity chromatography. A large variety of in vivo and in vitro labeling experiments have likewise shown that no incorporation of precursor aminoacid into receptors can be demonstrated.

These results indicate a discrepancy between the rate of expression of receptor binding activity and the rate of receptor synthesis as measured by aminoacid incorporation. Whether this reflects a true dissociation between biosynthesis and expression has yet to be verified. Although it appears unlikely that receptor sites are constitutive components of the cell membrane, such a proposal has previously been made about the development of gonadotropin receptors in the immature rat testis, and will be further explored in the ovarian system.

Significance.

The regulation of peptide hormone receptors in target tissues is now recognized as an important control mechanism for cell responses to hormone such as insulin and growth hormone. The control of gonadal responses to pituitary hormones is also regulated by alterations in receptor content and expression, and is an important factor in the endocrinology of reproductive processes.

Proposed Course.

The dependence of gonadal receptors on pituitary and steroid hormones will be analyzed in more detail, and biosynthetic studies will be pursued in the ovaries of PMSG/hCG-treated rats. A combination of immunofluorescence and binding techniques will be utilized to quantitate receptor occupancy and turnover during regression after hypophysectomy, and during 'down regulation' after treatment with exogenous gonadotropins.

Publications.

Hsueh, A.J.W., Dufau, M. L., Katz, S. I., and Catt, K. J. Gonadotropin Receptors: Immunofluorescence Labeling in Enzyme-Dispersed Interstitial Cells. Nature, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00152-01-RR

PERIOD COVERED

July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Radioligand-Receptor Assay of Gonadotropic Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	C. Williams, Ph.D., Staff Fellow, SHR, RRB, NICHD
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COOPERATING UNITS (if any)

LAB/BRANCH

Reproduction Research Branch

SECTION

Section on Hormonal Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.4	0.8

SUMMARY OF WORK (200 words or less - underline keywords)

The binding characteristics of gonadal receptor sites for LH and FSH were analyzed in particulate testis fractions by kinetic and equilibrium studies with radioiodinated gonadotropins. Computerized methods of analysis were applied to receptor binding data to permit accurate modeling of receptor-hormone interactions, and to incorporate the several correction factors which should be employed to derive binding constants for labile systems such as peptide hormone receptors. More sensitive and practical radioligand-receptor assays for LH/hCG and FSH were developed. These methods, together with a sensitive in vitro bioassay for LH, were used to evaluate the binding and activation properties of native and modified gonadotropins.

PHS-6040
(12-75)

Objectives.

To characterize the binding properties of particulate gonadotropin receptors in gonadal homogenates and membrane preparations. Also, to develop improved radioligand-receptor assays for LH/hCG and FSH, and to apply such methods to the assay of gonadotropin activity in vitro, and to structure-function studies in chemically modified gonadotropins.

Methods Employed.

Quantitative binding studies with labeled gonadotropins.
Radioligand-receptor assays of gonadotropins.
Affinity chromatography upon receptor sites.
Computer analysis and curve-fitting.
In vitro bioassay of LH.

Major Findings.

1) Radioligand-Receptor Assay of LH and hCG. The original method developed in this laboratory has been modified and improved to give an LH/hCG assay procedure of increased sensitivity and simplicity. The use of tracer ^{125}I-hCG, prepared by enzymatic iodination and group-specific affinity chromatography upon Sepharose-Concanavalin A has been combined with reduction of assay volume and of the quantity of LH receptor sites (rat testis homogenate) employed in the assay. Also, the use of rat testis total homogenate, rather than the 1500 g fraction of the homogenate, has been shown to give satisfactory assay conditions when the final separation of bound and free tracer is performed by centrifugation at 1500 g. Even though the original homogenate contains a proportion of binding particles which are not sedimented at 1500 g, the homogenate becomes sufficiently aggregated during the assay incubation that virtually all binding activity is sedimented at 1500 g after incubation of the assay suspension at 24°C for 16 hours.

These conditions for radioligand assay of LH/hCG have given a procedure of improved sensitivity, with half-maximum inhibition of binding by less than 1 nanogram of hCG, and detection limit of about 50 picograms hCG. In addition, the testis homogenate from one rat provides sufficient binding activity for about 2000 assay tubes with initial tracer binding of 15-20%. This method has been used routinely for radioligand-receptor assay of gonadotropin preparations, and for measurement of the specific activity of ^{125}I-labeled LH and hCG tracers employed for quantitative binding studies.

2) Characterization of Radioiodinated Gonadotropin Tracers. When valid binding constants are to be derived from kinetic or equilibrium binding studies with receptor preparations and labeled gonadotropins, it is essential to perform careful characterization of the specific activity and binding properties of the tracer hormone.

a) Measurement of specific activity. This is most appropriately performed in a biological assay system such as the radioligand receptor assay or the in vitro bioassay employing dispersed Leydig cells. The standard procedure has employed "self-displacement" assay of increasing amounts of ^{125}I-hCG in the testis radioligand assay, and comparison with standards of unlabeled hCG from the preparation employed for radioiodination. This method gives a measure of specific activity in terms of the biological activity of the hormone used for labeling.

b) Measurement of "maximum binding activity". This estimate is performed to determine the amount of biologically active hormone in the labeled preparation, by measurement of the proportion of tracer which can be bound during incubation with an excess of receptor sites. This factor should be used to correct the values for 'bound' and 'free' hormone derived during binding studies, to remove the error introduced by the inevitable presence of a proportion of inactive tracer in the labeled hormone preparation.

c) Furthermore, to calculate binding constants on a molar basis, the data should be corrected for the biological activity of the hormone employed for labeling, if this is not of maximum potency.

3) Kinetics of Gonadotropin Binding by Rat Testis Receptors. The rate constants of gonadotropin-receptor interaction in the testis were determined at 24°C and 37°C over a wide range of hormone concentrations. The kinetics of degradation of hormone and receptors were also determined at those temperatures, and incorporated into a computer analysis of the binding data by modifications of the differential equation for a second-order reaction. The association rate constant (Ka) was 4.7×10^7 M^{-1} min^{-1} at 24°C and 11×10^7 M^{-1} min^{-1} at 37°C; the corresponding initial dissociation rate constants (Kd) were 1.7×10^{-3} and 4.6×10^{-3} min^{-1}.

Reactant degradation was shown to have little effect upon measurement of the association rate constant, but caused overestimation of the dissociation rate constant derived from association experiments. Also, reactant degradation in equilibrium binding experiments caused an underestimate of the equilibrium association constant (Ka), but had less effect upon the determination of binding site concentrations. The effects of these factors could be corrected by independent measurements of the degradation processes, and the use of computer analysis employing the modified binding equation. In addition, a computer method was developed for analysis of equilibrium binding data derived by saturation or binding-inhibition studies, with incorporation of appropriate correction factors for specific activity and binding activity of the tracer hormone. This technique is being applied to all computations of binding data for testicular and ovarian receptors for LH, hCG and FSH.

4) Properties of FSH Receptors of Rat Testis. Tracer FSH of high binding activity was prepared by lactoperoxidase iodination of human FSH, followed by elution of bound tracer from testicular binding sites at low pH. The latter procedure gave a four-fold increase in the binding activity of ^{125}I-labeled FSH. Binding of FSH tracer to testis receptors from immature (15 day) rats was higher at 37°C than at 24°C, whereas the converse was observed with adult testes, suggesting that hormone degradation was less marked in the immature testis.

The ^{125}I-hFSH tracer and unlabeled hormone were shown to react identically with testis binding sites, and the equilibrium association constant of the FSH receptors for hFSH was 2 x 10^9 M^{-1}, about 10-fold lower than that of the interstitial cell receptors for LH and hCG. The association rate constant (Ka) for FSH binding showed marked temperature dependence, being 1.6 x 10^6 M^{-1} min^{-1} at 24°C, and 4.8 x 10^6 M^{-1} min^{-1} at 37°C. Dissociation of bound FSH from testis receptors was a multiexponential process, with initial dissociation rate constant (Kd) of 1.7 x 10^{-2} min^{-1} at 37°C. Thus, the binding constants of the FSH receptors in the testis tubule differ by about one order of magnitude from those of the LH/hCG receptors of the interstitial cell.

5) Radioligand-Receptor Assay of FSH. The binding-inhibition properties of a wide range of FSH preparations were investigated in a specific FSH radioligand assay employing rat testis homogenate and ^{125}I-hFSH. The minimum detectable dose of purified hFSH was 0.6 ng, and the mean 50% inhibition dose was 7 ng. The binding-inhibition curves of sheep and other animal FSH preparations were similar to those of human FSH standards, in contrast to the lower slopes of sub-primate luteinizing hormone observed in the rat testis-^{125}I-hCG radioligand assay for LH and chorionic gonadotropins. A small but consistent degree of cross-reaction by highly purified hCG in the FSH assay was observed, and is attributable to the recognized FSH-like activity of hCG at high concentrations. Also, purified TSH showed a small but significant degree of cross-reaction, possibly attributable to the similarities in sequence between the beta subunit of FSH and TSH.

6) Radioligand Assay and In Vitro Bioassay of Native and Modified Gonadotropins. Each of these assays developed in the Reproduction Research Branch has been applied to the evaluation of biological activity in a wide variety of gonadotropin preparations and derivatives. Previously, the isolated subunits of LH and hCG were shown to be devoid of binding activity and biological actions, and desialylated glycoprotein hormones were shown to possess full or enhanced binding activity which contrasted with their low biological activity in vivo. Further studies showed that the asialo- and asialo-agalacto- derivatives of hCG act as full agonists in terms of steroid production in vitro, but are partial agonists for cyclic AMP production. Various derivatives prepared by chemical modifications of sheep LH (Papkoff) showed radioligand assay potencies similar to their biological activities measured in vitro, indicating the biological specificity of the receptor binding assay.

Comparisons of crude and purified sheep FSH and PMSG were also performed, showing marked increase of receptor binding potency with hormone purification. In addition, highly purified PMSG (Papkoff) was shown to possess extremely high potency in both FSH and LH radioligand assays, as well as in the in vitro bioassay for LH. Thus, both FSH and LH activities of PMSG are proportionally enhanced during purification of the crude hormone, reflecting the hybrid biological nature of the PMSG molecule.

Significance.

The development of radioligand-receptor assays and in vitro bioassay of gonadotropins during this program has provided useful new methods for the measurement of LH and hCG. These procedures are much more sensitive than conventional bioassays, and provide a more valid measure of gonadotropins than that obtained by radioimmunoassay. These methods are also of value for studies on the structural determinants of gonadotropic activity, and for in vitro analysis of factors which modulate hormone-receptor interactions.

Proposed Course.

Applications of radioligand-receptor assays to assay of gonadotropins in plasma and urine. More detailed measurements of temperature-dependence and thermodynamic properties of gonadotropin receptors of testis and ovary Examination of the structural basis of intrinsic cross-reaction between purified glycoprotein hormones (FSH, TSH, hCG) in receptor binding assays. More detailed analysis of the role of carbohydrate groups in the binding and activation properties of gonadotropic hormones.

Publications.

* Catt, K. J., and Dufau, M. L.: Methods for determination of gonadotropic in vitro: Solid-phase radioimmunoassay, radioligand-receptor assay and in vitro bioassay. In Crosignani, P. and James, V.H.T. (eds.), Recent Progress in Reproductive Endocrinology, London, Academic Press, 1974, pp. 131-162.

Ketelslegers, J.-M., Knott, G. D., and Catt, K. J.: Kinetics of gonadotropin binding by receptors of the rat testis. Analysis by a non-linear curve-fitting method. Biochemistry 14: 3075-3083, 1975.

Catt, K. J., Ketelslegers, J.-M., and Dufau, M. L.: Receptors for gonadotropic hormones. In Blecher, M. (ed.), Hormone Receptors, Marcel Dekker, in press.

Catt, K. J.: Solid-phase radioassay techniques. In Antoniades, H. (ed.), Hormones in Human Plasma, Harvard University Press, in press.

* Not previously included in annual report.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00154-01-RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Structure-Function Relationships of the Biological Activities of hCG.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI G. T. Ross, M.D., Ph.D., Chief, RRB, NICHD
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COOPERATING UNITS (if any)
Columbia University
Mayo Clinic

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	4	1

SUMMARY OF WORK (200 words or less - underline keywords)

This project is a component of a larger study of the immunological, biological and physical correlates of the structure of human chorionic gonadotropin, a peptide hormone secreted by normal and neoplastic trophoblasts and used as indicator of pregnancy and trophoblastic tumors. This hormone assayed in vivo appears to possess Thyroid Stimulating (TSA), Follicle Stimulating (FSA) and Interstitial Stimulating Activities, and these studies are directed toward determining the molecular basis of these activities.

PHS-6040
(12-75)

Objective.

To determine the molecular basis of the follicle stimulating activity (FSA) of human chorionic gonadotropin; is this an intrinsic property of the same molecule as that having interstitial cell stimulating activity (ICSA)?

In a collaborative study done with R. E. Canfield of Columbia University and A. Albert of the Mayo Clinic, crude human chorionic gonadotropin was subjected to gel filtration on a large Sephadex column. Aliquots of 12 pooled fractions of OD_{280} active material were assayed by the "biological fingerprint method" devised by Albert using immature female rats and ICSA was measured by the ventral prostate weight method.

Structure-function relationships of the biological activities of hCG homologous and heterologous preparations of complementary subunits of purified hCG and purified ovine LH (oLH) were used to produce biologically active recombinant molecules which were assayed immunologically and biologically.

Major Findings:

No evidence consistent with any change in FSA:ICSA activity was obtained among the fractions recovered by gel filtration, suggesting that these two activities are properties of molecules with very similar, if not identical physical properties.

The FSA of hCG was found to be lost during dissociation with subunits and to be recovered to the same extent as the interstitial cell stimulating and thyroid stimulating activities following recombination of subunits. These data suggest that these activities depend upon a common beta subunit at least, and possibly a common alpha subunit as well. To test this hypothesis of dependence upon a common beta subunit further, hybrids produced by combining ovine LH alpha subunits with hCG beta subunits were purified and tested for ICSA and FSA in _in vivo_ and _in vitro_ bioassays, receptor assays and radioimmunoassays. Preliminary results indicate that the hybrids have LH activity, indicating that recombination occurred.

Significance:

These data provide further circumstantial evidence consistent with the hypothesis that the FSA and ICSA of hCG are intrinsic properties of a single molecule. The experiment with the oLH-hCG hybrids provides a basis for determining if oLH contains FSA, since according to current dogma this activity should be intrinsic to the beta, not the alpha, subunit of either hCG or oLH.

Proposed Course:

These recombinations will be tested for FSH activity _in vitro_ and _in vivo_ and ratios of FSA to ICSA activities compared with those for hCG alpha - beta recombinants. This increased sensitivity of the _in vitro_ assays should permit us to determine whether oLH has intrinsic FSA.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00155-01 RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Hormonal Control of Mammalian Follicular Maturation and Oogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI G. T. Ross, M.D., Ph.D., Chief, RRB, NICHD
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S. Hillier, Ph.D., Visiting Fellow, RRB, NICHD
J. P. Louvet, M.D., Guest Worker, RRB, NICHD
A. Ayala, M.D., Visiting Fellow, RRB, NICHD
P. Mueller, M.D., Staff Fellow, RRB, NICHD
S. M. Harman, M.D., Gerontology Research Ctr., NICHD

COOPERATING UNITS (if any)

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7	6	1

SUMMARY OF WORK (200 words or less - underline keywords)

The role of intraovarian sex hormone in mediating gonadotrophic stimulation of follicular maturation, including atresia and oogenesis has been under study in this laboratory for the past five years. These studies indicate that estrogens stimulate while androgens inhibit granulosa cell proliferation and enhance atresia during preantral stages of follicular maturation. Since ovarian biosynthesis of androgen requires LH stimulation, but biosynthesis of estradiol requires FSH stimulation as well, the role of gonadotrophins in stimulating both growth and atresia is mediated by sex steroid hormone synthesis.

PHS-6040
(12-75)

Objectives.

These studies are directed toward understanding the role of gonadotropins and sex steroid hormones in initiating and regulating follicular maturation leading to atresia and ovulation in mammalian ovaries. The observation that endogenous androgens stimulate atresia in ovaries of estrogen-primed hypophysectomized immature female rats has been pursued.

Methods Employed and Findings.

A substance with the properties of an androgen receptor in cytosol of granulosa cells from preantral follicles has been characterized further. The molecule has been shown to have a Stokes radius of 73 Å with a ratio of 1:9, and an estimated molecular weight of 24,000 Daltons. Activity is lost after treatment with n-methyl maleimide.

Putative receptors appear to be required for nuclear translocation of the hormone in steroid hormone target tissues. Pulsing whole animals or incubating minced ovarian tissue with ^{3}H-testosterone has been shown to result in translocation of the androgen to the nuclei of granulosa cells from preantral follicles. The hormone extracted is bound to a macromolecule with a sedimentation velocity of 5 to 5.5 on sucrose density gradients. Nuclear translocation of testosterone is consistent with a role of the steroid in regulating growth and atresia.

By analogy to actions in other tissues, an increase in RNA polymerase II activity is an early event accompanying nuclear translocation of steroid hormones. Furthermore, in rat uterus, a new species of protein appears following estrogen stimulation. Studies are in progress to determine if similar effects can be shown in granulosa cells for preantral rat ovarian follicles exposed to testosterone.

Transformation of preantral follicles to graafian follicles with antra occurs when estrogen-primed hypophysectomized immature female rats are given Follicle Stimulating Hormone (FSH). The biochemical events accompanying this process have not been systematically studied. The observation that $^{35}SO_4$ was incorporated into mucopolysaccharides in antral fluid from ovarian follicles of rabbits pulsed with $^{35}SO_4$ led us to ask if this process might be stimulated during antrum formation in ovaries of estrogen-primed hypophysectomized immature female rats receiving FSH. Incorporation of the label was shown to be proportional to the dose of FSH given. Neither interstitial cell stimulation nor androgen synthesis appeared to be required for this effect in estrogen-primed rats.

Estrogen dependence of this process is currently under evaluacion. Studies will be extended to determine whether FSH stimulates synthesis of mucopolysaccharides in addition to enhancing sulfate incorporation.

Significance:

An intraovarian site of action of sex steroid hormone control of ovulation constitutes another locus with potential for voluntary reversible control of mammalian ovulation. Such studies are consistent with the Institute's mission in generating the scientific substrate required in developing methods for voluntary fertility control.

Publications.

Harman, Mitchell S., Louvet, Jean-Pierre, and Ross, G. T. Interaction of estrogen and gonadotrophins on follicular atresia. Endocrinology 96: 1145, 1975.

Louvet, Jean-Pierre, Harman, Mitchell S., and Ross, G. T. Effects of human chorionic gonadotropin, human interstitial cell stimulating hormone and human follicle-stimulating hormone on ovarian weights in estrogen-primed hypophysectomized immature female rats. Endocrinology 96: 1179, 1975.

Harman, Mitchell S., Louvet, Jean-Pierre, and Ross, G. T. Inhibition of the ovarian augmentation reaction by a chemical antiestrogen. Endocrinology 96: 1119, 1975.

Louvet, Jean-Pierre, Harman, Mitchell S., Schreiber, James, and Ross, G. T. Evidence for a role of androgens in follicular maturation. Endocrinology 97: 336, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00156-01-RR

PERIOD COVERED

July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Anorexia Nervosa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	D. Lynn Loriaux, M.D., Head, Endocrinology Service Unit, RRB, NICHD
Other	R. Vigersky, M.D., Clinical Associate, RRB, NICHD

COOPERATING UNITS (if any)

LAB/BRANCH

Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: .5	PROFESSIONAL: .5	OTHER:

SUMMARY OF WORK (200 words or less - underline keywords)

This project has attempted to delineate organic or non-psychiatric abnormalities associated with Anorexia Nervosa. The primary area of investigation has been hypothalamic function, and the possible role of hypothalamic dysfunction in the pathophysiology of anorexia nervosa.

PHS-6040

Objectives:

Anorexia Nervosa has clasically been considered as a psychiatric disorder. A number of the features of this disorder, however, are compatible with a hypothalamic lesion. These include cold intolerance, amenorrhea, disordered satiety, and mood and sleep disturbances. The objective of this study has been to quantitate some of these parameters of hypothalamic function in both normal and anorexic subjects and to attempt to define their role in the pathophysiology of the disorder.

Methods Employed:

The only technology unique to the study has been that used to quantitate core temperature regulation in these patients in response to thermal stress. This entails the use of an environmental chamber to establish hot and cold stress while core temperature is continually monitored via a rectal thermistor. This chamber has been constructed in the 10B corridor.

Major Findings:

We have previously shown that patients with anorexia nervosa have hypogonadotropic-hypogonadism, partial diabetes insipidus and core temperature instability under thermal stress. This year we have shown that anorexic patients have a quantitatively normal, but qualitatively abnormal response to the hypothalamic releasing factors. This response is characteristic of hypothalamic dysfunction seen with anatomic lesions in this region, e.g., craniopharyngioma. A double blind treatment protocol attempting to treat these patients with cyproheptadine, an antiserotonergic drug, is nearing completion.

Significance:

These studies suggest that anorexia nervosa may be a primary hypothalamic disorder and not be of psychogenic etiology. If this is indeed the case, it suggests that perhaps a treatment may be found other than psychiatric, a treatment mode having a low success rate.

Projected Course:

The double blind treatment protocol will be completed in the coming year and this project will be concluded.

Publications.

Vigersky, R., Loriaux, D. L., Andersen, A., and Lipsett, M. B., Anorexia Nervosa, in Clinics in Endocrinology and Metabolism (M. Albrink, ed.), Blakiston Publ., Co., London, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00157-01-RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
Physiology of Puberty

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	D. Lynn Loriaux, M.D., Head, Endocrin. Service Unit, RRB, NICHD, NIH
Other	G. Cutler, M.D., Clinical Associate, RRB, NICHD, NIH

COOPERATING UNITS (if any)

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to gain insight into the physiologic processes underlying normal and abnormal pubertal progression. Principal areas of investigation include the mechanism of adrenarche and the temporal changes in hypothalamic function with respect to gonadotropin regulation.

PHS-6040
(12-75)

Objective:

This is a new project started this year. Although puberty is a nearly universal process in man, the underlying physiology of even the most fundamental aspects of this process are poorly understood. The objective of this study is to gain insight into the molecular physiology of two aspects of puberty; adrenarche and hypothalamic gonadotropin regulation.

Methods Employed:

Current investigations are confined to the adrenarchal process. A model system has been developed using the guinea pig, a rodent that undergoes adrenarche much like man in that DHA is the adrenal secretory product which serves as a marker for the process. Changes in adrenal physiology are monitored using a number of techniques, including solid phase receptor assay and steroid radioimmunoassay for A4 and A5 adrenal steroids.

Major Findings:

Clinical observation suggests that maturation of the adrenal, adrenarche, depends upon a gonadal signal. It is likely that this signal is either estrogen or androgen in nature. We have identified estradiol cytosol receptors in the guinea pig adrenal gland identical to those found in the rat uterus. No androgen receptors have yet been found.

Significance:

This finding supports the hypothesis that gonadal estrogen initiates or supports androgen production by the adrenal gland, a process beginning at the time of adrenarche.

Course:

In the coming year we will attempt to identify the cellular biosynthetic response of the adrenal gland to gonadal estrogen and investigate the role of this response in the adrenarchal process. We would hypothesize that an enzyme or a series of enzymes are induced, the most likely among these being 17-hydroxylase and the 17-20 desmolase, leading to increased A4 and A5 androgen production by the adrenal cortex.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00158-01-RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Steroid Mechanism of Action

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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S. Rifka, M.D., Visiting Fellow, RRB, NICHD, NIH

COOPERATING UNITS (if any)

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to provide information basic to the understanding of disordered phenotypic secondary sexual expression, including such disorders as gynecomastia and hirsutism. Principal areas of investigation include steroid production rates and metabolic disposition, plasma steroid transport and end organ steroid-receptor interaction.

PHS-6040
(12-75)

Objectives:

Abnormal sexual phenotypic expression presumably always relates to a distorted steroid sex hormone relationship at some point in development. This disordered relationship can be the result of an abnormality at least three loci: 1) sex steroid production, metabolism and clearance; 2) sex steroid plasma transport state and 3) end organ receptor abnormalities. The objective of this study is to clarify, at each of these three levels, the pathophysiology of two common secondary sex phenotypic disorders, gynecomastia and hirsutism.

Methods Employed:

Methods used in these studies encompass a wide variety of chemical and laboratory tools including RIA, radioreceptor assay, protein and enzyme analysis and the more commonly applied methods of clinical investigation, such as metabolic balance studies, histology and roentgenography.

Major Findings:

There are two main causes of drug induced gynecomastia; spironolactone therapy and digitalis therapy. We have clarified the mechanism of each. Spironolactone acts as an antiandrogen at the end organ, while digitoxin acts as a weak estrogen of the end organ. Both actions result in an increased relative estrogen exposure and this results in breast development.

Significance:

The fact that two such commonly used drugs can interact with estrogen and androgen receptors has great therapeutic importance. First, it explains the untoward side-effects of these drugs, in particular, gynecomastia. Spironolactone has been implicated in the development of carcinoma of the breast, and these findings lend a rational basis to that association. Additionally, therapeutic rationale is provided for the avoidance of these two drugs in estrogen responsive cancers, e.g., carcinoma of the breast.

Proposed Course:

In the coming year we will attempt to clarify the pathophysiology of alpha-methyl dopa and reserpine induced gynecomastia using the same model. A clinical study of three common types of gynecomastia; pubertal, senescent and idiopathic or the stable adult form, will continue.

Publications:

1. Van Thiel, D. H., Gamler, J., Lester, R., Loriaux, D. L., and Braunstein, G. D., Plasma Estrone, Prolactin, Neuroplasm and SHBG in Chronic Alcoholic Men, Metabolism 24: 1015, 1975.

2. Stripp, B., Taylor, A. A., Bartter, F. C., Gillette, J. R., Loriaux, D. L., and Easley, R. B., The Effect of Spironolactone on Sex Hormones in Man. J. Clin. Endocrinol. Metab. 41: 777, 1975.

3. Pita, J., Lippman, M., Thompson, B., and Loriaux, D. L., Interaction of Spironolactone and Digitalis with the 5 α DHT Receptor of Rat Ventral Prostate. Endocrinology 97: 1521, 1975.

4. Redman, E., Murphy, D. H., and Loriaux, D. L., Plasma Sex Steroid in Free Ranging Monkeys: Correlations with Social States, Am. J. Psych., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00159 01-RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)
CNS-Pituitary-Gonadal Interaction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	D. Lynn Loriaux, M.D., Head. Endocrin. Service Unit, RRB, NICHD, NIH
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	S. Marynick, M. D., Clinical Associate, RRB, NICHD, NIH
	S. Winters, M. D., Clinical Associate, RRB, NICHD, NIH
	E. Goren, M. D., Clinical Associate, RRB, NICHD
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COOPERATING UNITS (if any)

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 2	PROFESSIONAL: 2	OTHER:

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to understand the efferent and afferent limbs of the CNS-pituitary-testicular feedback system. Principal areas of investigation are LH-Leydig cell interaction, intratesticular steroid biosynthetic autoregulatory systems, and testicular modulation of LH and FSH secretion.

PHS-6040
(12-75)

Objective:

The objective of this project is to gain a better understanding of both the hypothalamic-pituitary-Leydig cell interaction and the Leydig cell-hypothalamic-pituitary feedback system. The hormonal effects of LH, FSH, estradiol, testosterone and dihydrotestosterone within the testis are also under investigation.

Methods Employed:

The methods employed vary with the study, but these generally include RIA, radioreceptor assay, PAGE and equilibrium dialysis.

Major Findings:

Four principal findings emerged this year:

1) The androgen binding protein that is found in rodents and which appears to be a Sertoli cell marker in that species does not exist in primates.

2) The plasma sex hormone binding globulin in primates is concentrated in ovarian cysts and normal follicles. This does not occur in rodents.

3) The feedback of the testes to the hypothalamus depends, in the greater part, on estradiol and not testosterone. A pure androgen effect, however, has been identified in the feedback system.

4) The blood-CSF barrier can serve as a potential control point in the feedback between testes and hypothalamus and differentially admits certain steroids to the CSF while excluding others.

Significance:

The role of estradiol in the feedback control of LH secretion in males is a new concept and challenges our current thinking in this area. The role of the blood-CSF barrier in selectively admitting certain steroids and, for practical purposes, excluding others, is similarly a new concept and has served to explain the weak LH suppressive potency of DHT compared to testosterone. The finding that ABP is not identifiable in primates renders it of little interest in human spermiogenesis.

Proposed Course:

In the coming year, we intend to explore the feedback effects of the important testicular estrogens and androgens on the isolated hypothalamus and isolated pituitary in the rhesus monkey. This is intended to isolate the locus of feedback activity of these two classes of sex steroids. The effects of locally produced estradiol on Leydig cell function is under investigation, as is the effect of prolactin on the LH-Leydig cell interaction.

Publications.

1. Vigersky, R. A., Easley, R. B., and Loriaux, D. L., Halotestin and Its Action on Gonadotropins in Man. J. Clin. Endocrinol. Metab., in press.

2. Vigersky, R. A., Loriaux, D. L., and Lipsett, M. B., Androgen Binding Protein of Testis, Epididymis and Plasma in Man and Monkey, J. Clin. Invest., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00160-01-RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies of the Biology and Molecular Events in Steroid Biosynthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	C. A. Strott, M.D., Senior Investigator, RRB, NICHD
Other	P. Ray, Visiting Fellow, RRB, NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

The long-term aim of this project is to understand in greater depth the biology of the steroid-synthesizing cell. There are several areas of uncertainty as to the exact molecular events involved in 1) initiation of steroid synthesis, 2) subcellular, intercompartmental transport of steroid precursors and intermediates and, 3) secretion of steroid products. In addition, the adrenal cortex is zonated with the outer zone cells synthesizing aldosterone (mineralocorticoid) and the inner zone cells synthesizing cortisol (glucocorticoid) and dehydroepiandrosterone (androgen). Yet, nothing is known of the cell-cell (zone-zone) interaction of the various zones. Current areas of research involve: 1) an examination of the role of microtubules and contractile proteins in steroid synthesis; 2) identification and characterization of non-catalytic proteins (membrane bound and/or soluble) that interact specifically with steroid precursors and intermediates; 3) measurement of subcellular pool sizes of cholesterol and pregnenolone under various conditions; 4) establishing the methodology for performing superfusion studies and, 5) morphological studies utilizing the electron microscope.

PHS-6040

A. Microtubules and Tubulin

Microtubules are subcellular polymeric organelles which are composed of subunits of a protein dimer called tubulin. The polymeric or microtubule form is in a dynamic state of equilibrium with the dimeric or tubulin form. It has been shown with an ACTH-dependent rat adrenocortical tumor cell line in tissue culture that agents which prevent tubulin from polymerizing into microtubules will stimulate steroid synthesis to the same magnitude that ACTH itself will. Several questions come to mind: 1) What is the nature of the molecular events that control and regulate, at any given moment, the dimeric polymeric equilibrium state of tubulin? 2) Since tubulin is a ubiquitous protein found in many different cell types (e.g. brain tissue) while steroid synthesis is confined to specific cell type (e.g. adrenal cortex), is there any intrinsic difference between the tubulins of the adrenal cortex and brain? 3) Is there a quantitative correlation between tubulin content of the steroid-synthesizing cell and steroid production? 4) Finally, can the experiments performed with a tumor cell line in tissue culture be confirmed with normal, intact tissue?

During this fiscal year, P. Ray has arrived on a Visiting Fellowship and she has been devoting her full efforts in this area. She has developed a technique for extracting and partially purifying tubulin and performed numerous studies to optimize conditions for a quantitative analysis. In addition, she has worked out a polyacrylamide gel electrophoretic system for a comparative analysis of tubulin extracted from the adrenal cortex of six different species, including man. These data have been further compared to data she has obtained on tubulin extracted from brain tissue of several species. The results of these many studies are being readied for publication.

Currently Dr. Ray is developing a superfusion technique to further study the role of microtubules in the control and regulation of steroid synthesis by the adrenal and gonad. It is also planned to examine contractile proteins under similar conditions.

B. Non-Catalytic Steroid-Binding Proteins.

1. Cholesterol

The rate-limiting step in steroid synthesis is considered to be the conversion of cholesterol to pregnenolone (viz. the cholesterol lyase reaction). This reaction occurs at an intramitochondrial site (possibly associated with the outer membrane). Cholesterol is an absolute requirement for steroid synthesis. This sterol is ubiquitous, being found in membranes, stored esterified to fatty acids in cytoplasmic lipid droplets; it can also be made available through de novo synthesis and extraction from the circulation. The exact location and pool size of cholesterol utilized in steroid synthesis are not known. It is presumed, however, that the principal source of cholesterol is extramitochondrial. If this is true then it must move into the mitochondria where the cholesterol lyase is located. The molecular events involved in this transport are not known.

2. Pregnenolone.

Pregnenolone is the product of the rate-limiting step in steroid synthesis (i.e., the cholesterol lyase reaction). One theory of regulation of steroid synthesis involves the concept of product inhibition, that is, as the local concentration of pregnenolone increases the lyase reaction is slowed and vice versa. True or not, the pregnenolone which is formed at a specific intramitochondrial site must move to specific extramitochondrial sites for further enzymatic alteration on its way to becoming cortisol, aldosterone, testosterone and estradiol. The molecular events involved in this transport are also not known.

It is conceivable that with both cholesterol and pregnenolone specific proteins acting as transport vehicles are involved. We have been investigating this possibility and have discovered two proteins, one heat stable and one heat labile. The heat stable protein interacts specifically with cholesterol and cholesterol sulfate. The heat labile protein interacts specifically with pregnenolone and pregnenolone sulfate. To date, our work has been confined to the soluble cell fraction and the data are currently being prepared for publication. In the future we will be developing techniques to look at organelle associated proteins. We are interested in their synthesis, turnover rate, physico-chemical characteristics, specific localization sites, and functional status.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00161-01 RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Role of Sex Steroids in Regulation of FSH and LH Levels in Men

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	R. J. Sherins, M.D., Senior Investigator, RRB, NICHD, NIH
Other	D. L. Loriaux, M.D., Ph.D., Senior Investigator, RRB, NICHD, NIH
	J. J. Janick, M.D., Clinical Associate, RRB, NICHD, NIH
	S. P. Marynick, M.D., Clinical Associate, RRB, NICHD, NIH

COOPERATING UNITS (if any)

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of the administration of testosterone and estradiol to normal men at physiological dosage demonstrate that estradiol, which is produced primarily by peripheral conversion of testosterone, accounts for the majority of the suppressive effects of testosterone on LH and FSH secretion. Blockade of estradiol action, by use of the antiestrogen, clomiphene, completely reverses the suppressive effects of testosterone on plasma gonadotropin concentrations. The data emphasize the important role of peripheral aromatization of testosterone in its physiological interaction in the hypothalamus and pituitary.

Objectives.

A considerable body of data exists which suggests that androgens regulate pituitary LH secretion in men by negative feedback inhibition. Estradiol, however, also significantly suppresses LH secretion. Since estradiol is produced primarily from testosterone by aromatization in peripheral tissue, any study of the role of testosterone per se on gonadotropin secretion measures the effect of both testosterone and estradiol. Accordingly, a series of studies have been developed to assess the relative contributions of testosterone and estradiol to the regulation of FSH and LH concentrations in normal men.

Methods Employed.

Testosterone and estradiol were administered to normal men at physiological dosage by constant intravenous infusion in order to mimic endogenous testicular sex steroid secretion. Multiple blood samples are obtained at 20 minute intervals during 8 hrs before and after 4 days of infusion to assess the suppressive effects of sex steroids on the mean concentration and pulsatile release of both gonadotropins. Additionally, an anti-estrogen, clomiphene, was administered to normal men prior to sex steroid infusion in order to observe whether testosterone would continue to exert its suppressive effect on gonadotropin levels during estrogen blockade.

Major Findings.

Testosterone, administered at mean production rate (7 mg/day) suppressed LH and FSH equally (30%) suggesting that there is no differential effect of this androgen on either gonadotropin. Infusion of estradiol at mean production rate (45 μg/day) produced similar suppression of FSH levels but slightly lesser decreases in LH (22%), demonstrating that the bulk of LH suppression by testosterone can be accounted for its estradiol equivalent. Administration of dihydrotestosterone, a potent androgenic metabolite of testosterone which cannot be peripherally aromatized to estrogen, was also effective in suppressing LH and FSH suggesting that there is a pure androgenic component to LH suppression as well. Thus, there appears to be an important role for both testosterone and estradiol in the feedback regulation of LH and FSH.

Clomiphene blockade, during seven weeks, completely blocked the suppressive effects of both estradiol (90 μg/day) and testosterone (15 mg/day) (which were infused at twice their normal production rates) further emphasizing the importance of aromatization of testosterone in regulation of gonadotropin secretion.

Significance.

These data add considerable insight into the mechanism of control of gonadotropin secretion in men and suggest that this regulation is far more complex than originally conceived. These studies are in keeping with the primary mission of the Branch in reproductive biology and emphasize the importance of the availability of human subjects provided by the Clinical Center.

Proposed Course.

1) Determining the effect of pure androgens, such as dihydrotestosterone, on gonadotropin secretion during clomiphene blockade.

2) Utilize inhibitors of peripheral aromatization to further assess the role of androgen to estrogen conversion in regulation of gonadotropin secretion.

Publications.

1. Loriaux, D. L., Vigersky, R. A., Marynick, S. P., Janick, J. J., and Sherins, R. J. Androgen and Estrogen Effects in the Regulation of LH in Man, in New Concepts of the Testis in Normal and Infertile Men: Morphology, Physiology and Pathology. P. Troen and H. Nankin (eds.), Raven Press, New York, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00162-01 RR

PERIOD COVERED
July 1, 1975 - June 30,1976

TITLE OF PROJECT (80 characters or less)
Studies of a Specific FSH Inhibitor from Seminiferous Tubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	R. J. Sherins, M.D., Senior Investigator, RRB, NICHD, NIH
Other	H. Wachslicht, M.D., Visiting Fellow, RRB, NICHD, NIH
	A. Chrambach, Ph.D., Senior Investigator, RRB, NICHD, NIH
	D. C. Klein, M. D., Senior Investigator, LBS, NICHD, NIH

COOPERATING UNITS (if any)

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Preliminary studies of aqueous extracts of rat testes demonstrate dose dependent suppression of FSH in castrate male rats. Variable suppression of LH is also produced but these effects appear not to be due to sex steroid contamination. These data support the hypothesis that seminiferous tubules produce a non-steroid FSH inhibitor (inhibin).

PHS-6040
(12-75)

Objectives.

Much indirect evidence has accumulated during the past five years, in both rodents and man, to suggest that there is a specific seminiferous tubular inhibitor of FSH secretion (inhibin), independent of the regulation exerted via sex steroids of Leydig cell origin. Very recently preliminary reports have appeared describing direct inhibition of FSH secretion by aqueous extracts of testis which are devoid of contaminating sex steroid. The purpose of this project is to develop a variety of methods to isolate and chemically characterize this putative inhibin.

Methods Employed.

Concentrates of aqueous extracts of rat testis are prepared using high speed supernatents of homogenized tissue, ultrafiltration of cytosol, organic solvent extraction and lyophilization.

These preparations were tested in acute and chronic castrated male rats to assess changes in gonadotropin concentrations.

Major Findings.

Aqueous extracts of whole rat testes or isolated rat seminiferous tubules produce consistent suppression of FSH and LH when administered to castrate immature male rats. This suppression appears to be dose dependent and not due to contaminating sex steroids. While suppression of FSH levels is consistent, LH suppression is variable.

Significance.

These data are provocative and suggest that testicular/pituitary interaction may indeed involve molecules other than sex steroids as classically described. Development of new concepts of FSH regulation is not only important in reproductive biology, but also may provide insight into mechanisms of altering reproductive function critical to such fields as contraception and infertility.

Proposed Course.

1) Characterization of gonadotropin suppression in castrate male rats to provide a reliable bioassay model for studies of FSH inhibitors.

2) Development of an in vitro bioassay model to facilitate studies of inhibin.

3) Development of fractionation techniques to isolate and characterize inhibin.

Publications.

1. Dixon, R. L., Lee, I. P. and Sherins, R. J. Methods to Assess Reproductive Effects of Environmental Chemicals: Studies of Cadmium and Boron Administered Orally. Environmental Health Perspectives, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00163-01 RR

PERIOD COVERED

July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Clinical Studies of Male Reproductive Disorders

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Sherins	Sr. Investigator	RRB	NICHD
Other:	W. Levis	Sr. Investigator	D	NCI
	N. Javadpour	Sr. Investigator	SURG	NCI
	P. M. Sternthal	Statistician	BB	NICHD

COOPERATING UNITS (if any)

Department of Anatomy, Harvard Medical School

LAB/BRANCH

Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

A variety of studies of clinical disorders of male reproduction are in progress. Currently we report 1) new information for the interpretation of semen analysis; 2) the first demonstration of an anatomical disorder of the Sertoli cell within the testis of infertile men; and 3) evidence for sperm-leukocyte interaction.

Objectives.

Our primary goal is directed toward understanding clinical reproductive biology with emphasis toward application of new information and methodology to diagnosis and treatment of the infertile male. To achieve our goal, it is imperative that we maintain both an outpatient diagnostic clinic and an in-patient hospital metabolic ward.

Methods Employed.

Several studies are in progress to develop insight into both etiology and treatment of the infertile male. All clinical physiology studies employ measurements of hormones (FSH, LH, Testosterone, Estradiol, etc.) in biological fluids as well as semen analysis.

A variety of other techniques have been developed or utilized in several specific projects. These include electron microscopy of testicular material from infertile men, ^{3}H-thymidine incorporation into sperm-leukocyte cultures and statistical-computer analysis of semen parameters of ejaculates collected from fertile and infertile men.

Major Findings.

1) Studies in collaboration with Dr. William Levis have shown that human sperm are capable of stimulating DNA synthesis in leukocytes of unrelated donors (allogeneic) but not from the sperm donor himself (autologous). Such DNA synthesis is seen when blood cells are exposed to antigens. These observations suggest that surface antigens on sperm may be important in understanding the immunobiology of reproduction and possibly some aspects of infertility.

2) It has become clear that 25% of the men referred to our clinic for infertility are, in fact, not infertile. The mis-diagnosis appears to be due both to misinterpretation of semen analysis prior to referral and to our uncovering of subtle gynecological abnormalities in the spouse. A prospective analysis of semen samples from over 100 men during the past four years has demonstrated that sperm counts and ejaculate quality of fertile men are considerably poorer than generally recognized, that 6-9 sequential semen samples may be required during 6 months to obtain a stable evaluation of semen quality and that, by discriminant analysis, sperm concentration and seminal cytology together are the best predictors of the infertile state.

3) Ultrastructural analysis of testicular biopsy tissue from men with a variety of disorders of spermatogenesis is in progress. The testes of men with germinal aplasia (Sertoli-only Syndrome) displays increased Sertoli cell vacuolization, markedly increased microtubular and filament formation in the basal compartment of the Sertoli cells and markedly increased Sertoli-Sertoli intercellular bridges. These findings are new and emphasize the importance of the supportive and nutritive role of the Sertoli cell to

spermatogenesis. This is the first anatomical evidence of a Sertoli cell disorder.

Significance.

The studies in progress and reported add measurably to our understanding of etiology of male reproductive disorders, provide insight into potential mechanisms of testicular disease and provide new information to facilitate the evaluation of the infertile male.

Proposed Course.

1) Pursue studies of human sperm induced leukocyte transformation as a model for study of cell mediated immunity in male infertility.

2) Use of low dose Pergonal in assessing the minimally effective dose of FSH required to initiate spermatogenesis in hypogonadotropic men.

3) Study a counter current exchange of testosterone from spermatic vein to spermatic artery in men and primates.

Publications.

1. Levis, W. R., Whalen, J. J., and Sherins, R. J. Mixed Sperm-Leukocyte Cultures as a Measure of Histocompatibility in Man. Science 191: 302-304, 1976.

2. Sherins, R. J. and Hodgen, G. D., Testicular Gamma-Glutamyl Transpeptidase: An Index of Sertoli Cell Function in Man. J. Reprod. Fertil., in press.

3. Sherins, R. J., Brightwell, D., and Sternthal, P. M., Longitudinal Analysis of Semen of Fertile and Infertile Men, in Concepts of the Testis in Normal and Infertile Men: Morphology, Physiology and Pathology. P. Troen and H. Nankin (eds.), Raven Press, New York, in press.

4. Chemes, H. E., Dym, M., Fawcett, D., Javadpour, N., Sherins, R. J. Ultrastructure of the Sertoli Cell in Men with Germinal Aplasia, in Concepts of the Testis in Normal and Infertile Men: Morphology, Physiology and Pathology. P. Troen and H. Nankin (eds.), Raven Press, New York, in press.

5. Sherins, R. J., Evaluation and Management of the Infertile Male. Syllabus of the Post Graduate Course, Annual Meeting of the American College of Ob-Gyn, Boston, Massachusetts, May 3, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00164-01-RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Protein Isolation by Electrophoresis Using An Objectively Defined Route

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	A. Chrambach, Ph.D., Senior Investigator, RRB, NICHD, NIH
Other	G. Baumann, M.D., Visiting Scientist, RRB, NICHD, NIH
	N. Y. Nguyen, Ph.D., Visiting Fellow, RRB, NICHD, NIH
	R. Houghten, Ph.D., Guest Worker, RRB, NICHD, NIH

COOPERATING UNITS (if any)
MIT, Cambridge, Massachusetts; U. of Calif., San Francisco, Calif.; Stanford U. School of Medicine, Palo Alto, Calif.; U. of Basel Medical School, Basel, Switzerland

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	

SUMMARY OF WORK (200 words or less - underline keywords)

A method for the preparative electrophoretic isolation of proteins up to gram levels ("isotachophoresis without spacers") was devised and applied to hGH-E. Electrofocusing was shown to be feasible in pH gradients made from buffers. Preparative gel electrofocusing was carried out without mechanical disruption of the gel by specific electrophoretic elution of zones. The first pieces of evidence were produced indicating a fundamental identity between "isoelectric focusing" and "steady-state stacking" (isotachophoresis). Analytical polyacrylamide gel electrophoresis (PAGE) in SDS-containing multiphasic buffer systems was optimized and extended to all systems operative at neutral and basic pH. Ampholine-protein interaction was shown not to be a problem in isoelectric focusing; a method for complete Ampholine removal from protein was developed. An electronic device for mobility measurement and an improved gel slicer were constructed, as was a device for PAGE with continuous optical scanning.

PHS-6040

4) Analytical Methods: a) Electrofocusing in buffer mixtures was developed replacing that in synthetic polymers ("Ampholine") which are limited in available pH ranges and costly. Besides economy, the method provides great flexibility in the making of pH gradients. This and other features of the method make it potentially attractive for application to two-dimensional mapping of multicomponent macromolecular systems. b) A gel type with improved properties, a method for Ampholine removal and evidence for the lack of chemical interaction between Ampholine and proteins in isoelectric focusing has been provided. c) An improved method of gel electrophoresis in the detergent, SDS, has been developed.

5) Theory: a) Although we do not as yet understand the causes of pH gradient instability in isoelectric focusing, we have increased the pool of known facts needed to elucidate the problem. b) We have produced the first evidence pointing to the essential identity between "steady-state stacking" and "electrofocusing". It involves interconversions between the two separation methods. Such interconversions have also practical relevance to multicomponent mapping. c) Resolution in a model system by isotachophoresis was investigated as a function of voltage and migration distance. d) Conformation of performance with theoretically predicted properties of isotachophoresis was studied.

Perspectives:

During the coming year, methodology of protein isolation and analysis will be pursued along four lines: 1) Biologically or medically important applications. The general methodology or "Quantitative PAGE" is now sufficiently developed for "export". The sole way to "export" methodology is to show that it works more efficiently, or in simpler fashion, than presently used other methods, when applied to "sexy" applications. Preparative isotachophoresis will be applied again, and multiply, to the isolation of enzymatically digested hGH-E. We will attempt to replace elution from gel slices by continuous electrophoretic elution. The second application will be hPRL from amniotic fluid. We will attempt the isolation of homogeneous isoenzymes for determination of specific activities in bioassay, RRA and RIA, and for sequencing in collaboration with Dr. Li, University of California.

2) Band width measurements. The continuous scanning device, presently operative in Dr. Catsimpoolas' laboratory, will be installed at NIH. Band width measurement during electrophoresis will be taken to determine diffusion coefficients, zone concentration in "stacks", to predict resolution in preparative electrophoresis. Since PAGE with continuous scanning provides very accurate mobilities after a few minutes of electrophoresis, and eliminates gel staining, photography, and measurements, it will in the long run become the routine analytical method, particularly in clinical chemistry requiring large numbers of daily analyses. Instrumentation development in this direction will be pursued in collaboration with Dr. Catsimpoolas.

3) Two-dimensional "macromolecular mapping". Present evidence shows that 12% of the proteins in E. coli can be mapped, using 2-D PAGE - separation on the basis of charge in one dimension, separation according to size in the other. We will be working during 1976-1977 on the technique and apparatus

development in that field with the purpose of providing a simple tool of tissue extract or body fluid analysis in biology and in the clinical laboratory. This is the field in which Dr. Nguyen is specializing.

4) Isotachophoresis with buffer spacers will be attempted preparatively. If successful, this technique should supplant the methodology presently applied to the isolation of hGH-E ("isotachophoresis without spacers").

Publications:

1. Baumann, G. and Chrambach A. Gram-preparative Protein Fractionation by Isotachophoresis: Isolation of Human Growth Hormone Isohormones. Proc. Nat. Acad. Sci. U.S., in press.
2. McCormick, A., Miles, L.E.M. and Chrambach, A. Selective Elution of Zones by Ampholytes or Buffers: A Method for Preparative Isoelectric Focusing on Polyacrylamide Gel. Anal. Biochem., in press.
3. Catsimpoolas, N., Griffith, A. L., Williams, J. M., Chrambach, A. and Rodbard, D. Electrophoresis with Continuous Scanning Densitometry: Separation of Cells in a Density Gradient. Anal. Biochem. 69: 372-384, 1975.
4. Tipton, H. W., Rumen, N. M. and Chrambach A. A Slicer for Polyacrylamide Gels of 3-, 6- and 18 mm Diameter. Anal. Biochem. 69: 323-326, 1975.
5. Chidakel, B. E., Baumann, G., Rodbard, D. and Chrambach A. A Simple Electronic Device for the Measurement of Band Migration Distances in Polyacrylamide Gels. Anal. Biochem. 66: 540-544, 1975.
6. Chrambach, A., Jovin, T. M., Svendsen, P. J. and Rodbard, D. Analytical and Preparative Polyacrylamide Gel Electrophoresis: An Objectively Defined Fractionation Route, Apparatus and Procedures, in Methods of Protein Separation: A Modern Survey (N. Catsimpoolas, ed.), Plenum Press, in press.
7. Chrambach, A. and Baumann, G. Isoelectric Focusing on Polyacrylamide Gel, in Isoelectric Focusing (N. Catsimpoolas, ed.), Academic Press, in press.
8. Nguyen, N. Y. and Chrambach, A. Non-isoelectric Focusing in Buffers, Anal. Biochem., in press.
9. Baumann, G. and Chrambach, A. A Highly Crosslinked, Transparent Polyacrylamide Gel with Improved Mechanical Stability for Use in Isoelectric Focusing and Isotachophoresis. Anal. Biochem. 7: 32-38, 1976.
10. Baumann, G. and Chrambach, A. Quantitative Removal of Carrier Ampholytes from Protein Fractions Derived from Isoelectric Focusing. Anal. Biochem. 69: 649-651, 1975.

11. Baumann, G. and Chrambach, A. Lack of Interaction Between Hormonal Proteins and Synthetic Carrier Ampholytes (Ampholine). Anal. Biochem. 64: 530, 1975.

12. Wyckoff, M., Rodbard, D. and Chrambach, A. Optimization of Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate (SDS-PAGE) Using Multiphasic Buffer Systems. Fed. Proc., in press.

13. Baumann, G. and Chrambach, A. Mechanism of pH-Gradient Instability in Isoelectric Focusing, in Progress in Isoelectric Focusing and Isotachophoresis (P. G. Righetti, ed.) Elsevier, Excerpta Medica, Amsterdam, 1975, pp. 13-23.

14. Chrambach, A., Baumann, G., McCormick, A., Miles, L.E.M. and Nguyen, N. Y. New Methods of Protein Isolation and Analysis by Gel Electrophoresis, in Proceedings of 10th International Congress of Biochemistry, Hamburg, Germany, in press.

ITHSONIAN SCIENCE INFORMATION EXCHANGE ROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00165-01-RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of Protein Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	A. Chrambach, Ph.D., Senior Investigator, RRB, NICHD, NIH
Other	G. Baumann, M.D., Visiting Scientist, RRB, NICHD, NIH
	M. Ben-David, Ph.D., Visiting Scientist, RRB, NICHD, NIH
	J. S. Skyler, M. D., Clinical Associate, NHLI, NIH
	A. R. Rogol, M.D., Clinical Associate, NIAMDD, NIH

COOPERATING UNITS (if any)
Hadassah Med. School, Hebrew University, Jerusalem, Israel; Duke University Medical Center, Durham, N. C.; U. of Virginia School of Med., Charlottesville, Virginia; U. of California Medical School, San Francisco, California

LAB/BRANCH
Reproduction Research Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

SUMMARY OF WORK (200 words or less - underline keywords)

Activated hGH isohormones D and E were isolated by a new method capable of producing homogeneous species in amounts sufficient for growth assay in man.

Human prolactin was prepared from amniotic fluid in 60% overall yield. From 10 l of the fluid, 3 mg of a mixture of hPRL isohormones A, B, C and D free from protein contaminants was isolated. Another 3 mg of hPRL was of lesser purity. The method, for the first time, provides milligram amounts of the hormone required for isohormone separation for biochemical and physical-chemical purposes.

I. Human Growth Hormone (hGH).

A. Biologically activated hGH. The purposes of this work on hGH-bioactivation are a) to provide the field of medicine with an effectively increased supply of hGH for therapy and b) to prepare homogeneous hGH-B (standard) and hGH-E (activated) for physiological studies and for chemical and physical-chemical investigation of the structure-function relationships.

We reported the 8-fold activation, in the rat tibia assay, of hGH partially digested by plasmin in 1970. In 1973, we published the isolation of four hGH isohormones, and their specific activities increasing to an 8-11 fold value from hGH-B to C to D to E. In 1975, we developed a high load capacity method for the separation of these isohormones ("isotachophoresis without spacers"). Therefore, we are now ready to manufacture hGH-E at the gram level required for testing of its specific bioactivity in man. This is just being started in collaboration with Drs. Blizzard and Levine. We have also distributed samples of hGH-B and hGH-E, properly coded, to Drs. Ellis and Grindeland for rat tibia assay, to Dr. Kostyo for rat weight gain assay and metabolic assays, to Dr. H. Friesen for testing in a hepatic radioreceptor assay, and to Dr. Lebovitz for somatomedin assay. The material has also been tested in Dr. Roth's lymphocyte binding assay (RRA) and by RIA. This study should result in a comprehensive bioactivity profile as the basis for the assay in man.

B. Physiological studies on hGH-E (carried out by G. Baumann, independently) The possible role of endogenous plasmatic plasmin in a conversion of hGH-B to hGH-E was investigated. Likewise, the role of peripheral tissues in the generation of hGH-E from hGH-B was tested in vivo in the Rhesus monkey model. Both of these studies gave no evidence for conversion in the circulation or in peripheral tissues. We, therefore, conclude that such a transformation could occur in the pituitary gland. Metabolic clearance rates of the isolated isohormones have been measured in man and Rhesus monkey. The preliminary data suggest that the clearance rate of hGH-D is slower by a factor of 2-3 than that of hGH-B. This observation may provide an explanation for their different activities.

C. An hGH catalog. hGH activity is associated with a large number of molecular species. Communication between laboratories, and increasingly the clinical administration of hGH, depends on a knowledge of "who's who". We have therefore begun a systematic physical characterization study on these various forms of hGH, starting with standard NPA preparations and some prepared by Dr. Li. We have also started to characterize synthetic fragments of hGH provided by Dr. Li.

D. Plasmin and subtilisin. The activation of hGH by plasmin digestion was attempted by four laboratories. Two succeeded, two didn't. The reason for the two failures was insufficient hydrolysis, probably due to inadequate enzyme. Also, the enzymes in the "winning" laboratories were crude, and different from each other. This prompted us to investigate the enzyme, its protein components and their specific abilities to produce hGH-E. Out of four enzymatically active groups of proteins within our preparation (Cutter), only one proved to do the trick. We will, during the next year, extend this study to a comparison with other available preparations of plasmin, as well as with the equally activating bacterial enzyme, subtilisin (a finding of U. J. Lewis), in order to be ready 1-2 years from now for the activation of the available pool of hGH with a well-characterized, maximally potent enzyme.

II. Human Prolactin.

The purposes for isolating hPRL from amniotic fluid are: a) to isolate the first homogeneous protein with hPRL activity for sequencing, b) to measure specific activity ratios on isolated hPRL isohormones (to test for differences in analogy with hGH), c) to make antibody against isolated isohormones, d) to prepare hPRL in amounts sufficient for chemical and physical-chemical studies (presently not available), e) to exemplify the use of Quantitative PAGE for protein isolation on a "needle in the haystack" isolation of a new hormone from a crude biological fluid.

a) Isolation from amniotic fluid. The isolation of a mixture of isohormones A, B, C and D of hPRL from amniotic fluid was improved procedurally to give a yield of 60%. Six milligrams of hPRL were made from 10 l amniotic fluid.

b) "Big hPRL". hPRL with low elution volume in gel filtration, considered "big" by widely held criteria, turns out to be of ordinary molecular size of hPRL and increased charge.

c) Charge properties. The methodology of molecular charge determination was exemplified on hPRL, and the persisting problems and uncertainties in the assessment of molecular net charge were summarized.

d) Iodinated. In biological studies and receptor studies of protein hormones, it is imperative to have an iodinated hormone which is conformationally indistinguishable from the uniodinated hormone. Using hPRL and hGH, we have demonstrated that unaltered iodinated hormones, by the criteria of "Quantitative PAGE", can be produced, using a lactoperoxidase procedure.

III. Testicular TeBG.

Human testis extracts contain a DHT-binding protein indistinguishable from plasma TeBG except that it exhibits polymorphism with regard to net charge and aggregation state, presumably due to higher concentrations of degrading enzymes and higher TeBG concentrations in the tissue compared to plasma.

IV. Ovarian cAMP Dependent Protein Kinase.

Three molecular forms of protein kinase, and three cAMP binding proteins have been characterized. The "classical" isolation method for this enzyme was shown to yield mostly contaminants. Isolation of the dominant kinase is in progress.

Perspectives.

During 1976-1977, it is planned to isolate hGH-E amounts required by the assay in man. hPRL from amniotic fluid will be fractionated with the purpose of isolating at least one isohormone in homogeneous form. The methodology of both hGH (isotachophoresis) and hPRL (isoelectric focusing) separation will be improved.

Publications.

1. Baumann, G. and Chrambach, A. Gram-preparative Protein Fractionation by Isotachophoresis: Isolation of Human Growth Hormone Isohormones. Proc. Nat. Acad. Sci. U.S., in press.

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3. Rogol, A. D., Ben-David, M., Sheats, R., Rodbard, D. and Chrambach, A. Charge Properties of Human Pituitary and Amniotic Fluid Prolactins. Endocrine Res. Comm. 2: 379-402, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00167-01-RR

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

[illegible] of Hormone and Neurotransmitter Release

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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J. M. Bonner	Sr. Staff Fellow	RRB	NICHD
C. Pazoles	Staff Fellow	RRB	NICHD
C. Creutz	Guest Worker	RRB	NICHD
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P. Chretien	Sr. Investigator	SURG	[illegible]
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BRANCH
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

SUMMARY OF WORK (200 words or less - underline keywords)

Release of hormones and transmitters from endocrine cells and nerve endings is believed to occur by exocytosis. While the molecular basis of this process remains obscure, it is known that isolated secretory vesicles from brain and adrenal medulla can be induced to release their contents by exposure to ATP, Mg^{++}, Ca^{++} and other ions. This reaction is viewed as a model of chemical events leading to vesicle fusion with the plasma membrane and subsequent fission. We have found in the adrenal that ATP analogues blocked at the β-γ position with -NH- or $-CH_2-$ can also induce release. These analogues are not cleaved by ATPase, and these results suggest that the motive force for release is not cleavage of ATP. We have also found that an intact α-β oxygen bond in ATP is required for release. Cleavage at this bond occurs in the adenyl cyclase reaction, and we have found this enzyme to be intrinsic to the vesicle membrane. Furthermore, it is activated only when the granule releases its contents. We conclude that ATP may be inducing release by acting as a cyclase substrate. In separate studies we have also found that isolated vesicles have a transmembrane potential, sensitive only to protons and ATP. ATP analogues that induce release can also induce depolarization of the vesicle. We conclude that depolarization of the vesicle as well as synthesis of cAMP, may be coupled to release.

Objectives:

1. The study of basic mechanisms of hormone and neurotransmitter release.
2. The molecular nature of signals which turn secretion "on" and "off".
3. Role of calcium and other ions in the regulation of secretion.
4. The comparative structures and interactions of secretory vesicles and plasma membranes.
5. Role of fibrous proteins such as myosin, actin and tubulin in secretion.
6. Isolation of proteins and glycoproteins involved in secretion.

Methods Employed:

1. Electron microscopy
2. Spectroscopy and fluorometry
3. Cell fractionation
4. Enzymatic analysis
5. Polyacrylamide gel electrophoresis
6. X-ray diffraction

Major Findings:

The following observations have been made on the bovine adrenal medulla, except as indicated.

1. The role of ATP analogues in ATP-induced release from chromaffin granules has been further studied. Analogues substituted at the β-α position, such as AppNHp and $AppCH_2p$ can induce release. However, while the K_m for ATP is 0.2 mM, the K_m for AppNHp is 0.8 mM. In addition, the pH optimum for ATP is 6.0-6.2, while the pH optimum for AppNHp is approximately 7.4. This is interpreted as being related to the higher pK_a for AppNHp. These results suggest that ATP induces release primarily by a mechanism unrelated to a traditional β-γ ATPase activity.

2. Adenylate cyclase has been found to be an intrinsic chromaffin granule membrane component. The enzyme co-purifies with dopamine-β-hydroxylase and cytochrome b562, both intrinsic granule components. The enzyme is activated by fluoride and GppNHp, and inhibited by β-agonists, such as isoproterenol, and nerve growth factor. Both β agonist and NGF inhibition are reversed by L-propranlol, a traditional β-antagonist.

3. The adenylate cyclase activity is also tightly coupled to ATP activated release. In intact granules, cyclase activity can be detected only in the presence of releasing conditions. This means the system must include Mg^{2+}, ATP and chloride. The magnitude of the cyclase response is directly proportional to the release rate.

4. We have recently found that adrenergic vesicles have a transmembrane potential which can be measured using the distribution of permeant ions such as methylamine and thiocyanate across the vesicle membrane. The potential is electrical in character, and is sensitive to protons but not other cations.

Mg^{++}-ATP has a profound effect on the potential, leading to a reversal of the sign. The $K_{1/2}$ for the ATP-induced depolarization is 40 μM, and the pH optimum is similar to that for ATP-evoked release. AppNHp, a structural analogue of ATP, can also depolarize the vesicle, as mentioned above, and can also provoke release from the intact vesicle.

5. The plasma membrane from the adrenal medulla has been isolated and compared with the purified chromaffin granule membrane. The two membranes have some qualitative similarities in that both contain large amounts of sialic acid and cholesterol. However, the plasma membrane fraction contains a number of unique protein bands when compared on SDS gels. Two of these proteins have been found to be tubulin and actin by analysis of tryptic peptides of iodinated protein bands. These proteins have been implicated in release of hormones and transmitters from cells, and their specific distribution on the plasma membrane may be of functional importance.

6. The superior cervical ganglion (SCG) of the rat has been studied in terms of the sensitivity of tyrosine hydroxylase (TH) induction to purified 7S nerve growth factor (NGF). Exposure of the ganglion to NGF results in an immediate burst of cAMP synthesis. The cAMP elevation lasts only 5 minutes. TH levels then begin to rise. These results demonstrate a kinetic relationship between cAMP synthesis and TH induction in the SCG.

7. Squid brain synaptosomes have been found to be sensitive to the psychoactive drug, chlorpromaxine. Uptake of monoamines (DA, NA, 5HT) but not other putative transmitters are affected.

8. Dopamine-β-hydroxylase (DBH) from chromaffin granules of the bovine adrenal medulla has been shown to have ion conductance properties when mixed with a black lipid membrane. Channel-forming properties are optimal at 100 mM KCl, and 4 mM $CaCl_2$ at pH 6.2. Dopamine, but not noradrenaline, activates the ionophoric properties of DBH.

9. Dopamine-β-hydroxylase (DBH) has been studied by electron microscopy using negative stains. DBH is found to form monomers, dimers and tetramers corresponding in size to multiples of the 75,000 MW subunit predicted from chemical studies.

Significance to Biomedical Research:

1. The study of isolated chromaffin granules has allowed us to develop a system for studying hormone or neurotransmitter secretion in a test tube. There are dysfunctions of this apparatus which may underlie important disease states. Examples include diabetes (adult diabetics have normal insulin levels in their pancreas); heart disease (norepinephrine is stored in chromaffin granules in cardiac nerves); schizophrenia (drugs which control behavior aspects of psychosis -- phenothiazines, reserpine - act directly on the chromaffin granule uptake system); mental retardation (very few anatomic bases for this syndrome have been detected; the defect may be at a more basic, synaptic level); pediatric hyperactivity syndrome (amphetamine therapy has a direct effect on brain chromaffin granules); Sudden Infant Death Syndrome (hypertrophy of adrenal medulla, secondary to chronic hypoxia is a common finding).

2. Cyclic nucleotides have been implicated as second messengers for many hormone systems. The nucleotide is believed to be generated by adenylate cyclases on the plasma membrane. We have shown that the chromaffin granule, a subcellular organelle, is also an important site for the enzyme in the adrenal medulla. This may also be the case for other secretory systems.

3. Isolated plasma membranes from adrenergic secretory cells have not hitherto been available for study. The fundamental problem of membrane fusion can now be studied directly and the mechanism of exocytosis elucidated.

4. Dopamine-β-hydroxylase has been identified as an important enzyme in neurotransmitter economy, and may be relevant to hypertension in humans. The identification of this enzyme as an ionophore is of great potential medical importance. The ionophoric character of DBH suggests that this protein could also function as a transmitter.

5. The development of the secretory machinery in the adrenal medulla may prove to be a prototype for secretion of all hormones and neurotransmitters.

6. Experiments on chlorpromazine effects on invertebrates show that the pharmacology of this compound is fundamental to even primitive nervous systems. The mechanism of drug action may be studied better in greatly simplified nervous systems.

Proposed Course:

1. Reconstitution of the process of secretion using components for the adrenal medulla system remains the major goal of the laboratory.

2. The effects of drugs such as desmethylimpramine, chlorpromazine and reserpine will be examined for their role in blocking uptake and release in granules. Receptors for these drugs will be sought in the granule and plasma membrane.

Publications.

1. Hoffman, P. G., Zinder, O., Nikodijevic, O., and Pollard, H. B., ATP-Stimulated Transmitter Release and Cyclic AMP Synthesis in Isolated Chromaffin Granules, J. Supramolec. Structure 4: 396, 1976.

2. Zinder, O., Nikodijevic, O., Hoffman, P. G., and Pollard, H. B., Adenylate Cyclase Activity in Purified Chromaffin Granule Membranes, J. Biol. Chem., 251: 2179, 1976.

3. Nikodijevic, B., Nikodijevic, O., Yu-M-Y. W., Pollard, H. B., and Guroff, G., The Effects of Nerve Growth Factor on Cyclic AMP Levels in Superior Cervical Ganglia of the Rat, PNAS (US) 72: 4769, 1976.

4. Nikodijevic, O., Nikodijevic, B., Zinder, O., Guroff, G., Yu, M-Y. W., and Pollard, H. B., Control of Adenylate Cyclase from Secretory Vesicle Membranes by Beta-Adrenergic Agents and Nerve Growth Factor, PNAS (US), in press.

NICHD ANNUAL REPORT
July 1, 1975 through June 30, 1976
Pregnancy Research Branch

SUMMARY

The Pregnancy Research Branch was eminently successful in pursuing its mission of investigating certain aspects of perinatal biochemistry and physiology in normal and abnormal mammalian pregnancy. No human research was performed. Rather, animal models of human pregnancy are used; these include the pregnant Rhesus monkey (Macaca mulatta), the pregnant baboon (Papio papio), and the pregnant Dorset sheep. The fact that data obtained from these preparations continues to equate to that which sporadically becomes available from human pregnancy strengthens and supports the belief that extrapolations from these animal models is pertinent to comments about human pregnancy.

There are two main sections in the branch. The Perinatal Physiology Section concerns itself both with the endocrine physiology of pregnancy as well as the cardiovascular dynamics of the placental circulation. We can now reliably achieve a surgically manipulated pregnant sheep preparation in a consistent manner. This research skill, a major advance which places the branch in the forefront of this type of research, consists of applying electromagnetic flow transducers on the common uterine artery of the pregnant horn and the intra-abdominal fetal umbilical vein in addition to cannulation of multiple vessels. The pregnancy is not interrupted and can be expected to continue for several weeks in a chronic nonstressed nonanesthetized state.

Because of the consistent reproduction of this preparation we have been able to increasingly examine those factors which influence both uterine and umbilical blood flow in the near-term pregnant sheep. The presence of beta and alpha adrenergic receptors in the placental circulation have been examined in various species. The presence of and the responsivity of fetal beta receptors in the sheep has been demonstrated by our use of isoproterenol to either mother or fetus. The ability to create a beta blockade was tested with the use of propranolol. When propranolol is infused, there is a modest increase in umbilical blood flow as well as fetal bradycardia. We also observed a decrement in umbilical blood flow with intravenous propranolol infusion to the mother. This occurred without significant change in maternal or fetal mean arterial pressure or uterine blood flow. It is not wise to extrapolate from the sheep to the human, but it is important to consider that clinicians have increasingly been suggesting the use of propranolol in the treatment of preeclampsia as well as in dysfunctional labor. There have been isolated reports of growth retarded infants with small placentas who were asphyxiated at birth of mothers who were given propranolol.

In this same area, there were extensive studies this year on the effects of beta adrenergic drugs on the placental circulation of the conscious pregnant sheep. We have specifically used as test drugs ritodrine, TH1165A, salbutamol, or isoxoprine because all of these agents are used increasingly by physicians in the treatment of premature labor and intrauterine growth retardation, and acutely with hyperactive labor. Our major finding is that infusion of these drugs to the ewe results in significant decreases in uterine blood flow and mean maternal arterial pressure. There were some differences between the agents, but these could reflect more the dose schedules we used rather than the difference in drug effect per se. We used drug dosages that equated to those that are reported in human literature. (This requires the unproven assumption that the metabolism of the drugs equates in both human and sheep and we have no knowledge of this. We are examining this latter point with the help of radioisotopically labelled drug.) During these infusions, umbilical blood flow and fetal heart rate either do not change or increase slightly with mean fetal arterial pressure remaining relatively constant. It is our belief that these agents must be evaluated over a prolonged infusion time to more accurately simulate therapy in the human. This is particularly pertinent because of the presence of end-infusion and post-infusion uterine blood flow increases allowing the suggestion that there is a possible accommodation by the uteroplacental vasculature to the drugs. Another extension of these studies was the examination of the effects of alpha agonists and alpha antagonists in this preparation. Phenoxybenzamine into either the mother or the fetus did not result in any significant changes in blood flows, arterial pressures, or heart rate. In contrast, methoxomine did produce a significant increase of arterial pressure, bradycardia, uterine blood flow decrease but no change in umbilical blood flow. Thus, we can confirm that alpha adrenergic receptors are both present and responsive in sheep mother and fetus near-term. It would also appear that alpha adrenergic stimulation, whether through stress or pharmacologic stimulation, can be potentially detrimental to the fetus. Again, there is a question if this information can be readily or should be extrapolated to humans. The net result of all of these studies does allow the suggestion that there is tonic beta adrenergic activity across the placental circulation and the possibility therefore exists that therapeutic measures can be directed toward improving inadequate flow.

Among the multiple influences on the placental circulation are those of the renin-angiotensin system. We have continued our investigations in this area by specifically examining the contributions of both the maternal and fetal kidneys to plasma renin activity levels in the respective circulations. It was our hope to study the effects of acute perturbations of maternal and fetal plasma electrolytes and volume on maternal and fetal plasma renin activity and electrolytes as well as to study the effects of a low sodium diet (still frequently inappropriately prescribed in this country to humans in pregnancy) on the same indices. Although the experiments are currently ongoing and all of the data has not been analyzed, it is now apparent that fetal postoperative survival in ewes on low sodium diets is markedly diminished compared to survival with normal sodium diets. We have been hampered in obtaining suitable data because of this problem and must now consider that a low sodium diet per se is a significant variable in our postoperative mortality.

Another set of variables for circulation are the prostaglandins, and we have begun preliminary studies directed toward the effects of prostaglandins and prostaglandin inhibitory agents on the sheep circulation. Again, it is known that prostaglandins are significant in maternal physiology and in the onset of labor; their direct effects on placental circulation has not been identified. This area will be the primary focus of our activities in the sheep model in the coming year. In preliminary investigations, we have found that the administration of PGE2 is associated with an increase in fetal blood pressure at small doses, and a decrease at large doses. PGF2α also results in an increase in fetal blood pressure and is associated also with an increase in fetal umbilical blood flow and fetal bradycardia. The effects of PGA will also be identified. We have three prostaglandin inhibitors (fenoprofen, naproxin, and indomethacin) available. We are presently examining the metabolic disposition of these agents so that we can be in a better position to understand the dose range that should be utilized. Our concern with animals is that differences in metabolism will result in either inadequate pharmacologic or supra-pharmacologic dosage if one is governed only by the human dose levels.

Many experiments are relatively short, and can be completed in a morning or afternoon. Some long experiments can last from morning through afternoon to evening. The presence of circadian rhythms becomes important in all of these instances. Circadian rhythms in the placental circulation of conscious near-term pregnant sheep have not been reported and we have specifically examined that issue this year. Circadian 24 hour period rhythms were found in uterine blood flow of all of the animals which were examined and smaller amplitude circadian rhythms were also found in umbilical blood flow. In no case were the rhythms synchronous with the light-dark cycle or was it synchronous between animals. However, consistent antisynchrony was always found between the maternal and fetal rhythms within a given animal. There was no consistent harmonic cycles in maternal arterial, fetal arterial, or amniotic fluid pressures. Therefore, these rhythms will need to be considered in prolonged experiments in order to delineate the effect of exogenous variables such as drugs or changes in environment.

Our long-term primary project in the pregnant Rhesus monkey has been to examine fetal carbohydrate metabolism. We took advantage of our experience with the beta adrenergic agents to examine the responsiveness of the normal fetal pancreatic alpha and beta cell to these agents. Antilabor drugs in humans is associated with glycogenolysis and elevations of plasma glucose in both mother and fetus. There is also evidence in human and other species that the beta adrenergic agents are associated with release of insulin from the beta cell. This was not the case in the normal monkey fetus, but was so when the fetus of a streptozotocin induced glucose intolerance pregnancy was examined. We also had an opportunity to preliminary examine the responsiveness of the fetal pancreatic beta cell in the glucose intolerant pregnancy when somatostatin was given with a glucose challenge. Somatostatin does not appear to inhibit the fetal insulin response to hyperglycemia and this differs markedly from consistent inhibitions by the hormone of the insulin response to a range of insulinogenic stimuli in adults of other species. Coupled with this research is the increasing awareness that the variables of surgery, anesthesia, and the associated stress, although superficially consistent from preparation to preparation, could adversely influence the studies we have done in carbohydrate metabolism. Further, there has been increasing success by three other groups using monkey preparations in obtaining a chronic model. This has required the use of either beta-mimetic agents or prostaglandin inhibitory agents. We have had previous experience with chronic restraint of pregnant baboons who were not surgically manipulated. We now believe that we have sufficient knowledge of the beta-mimetic agents and some knowledge of the prostaglandin inhibitors as well as an appropriate restraining chair to begin a significant effort in obtaining chronic restraint of a near-term pregnant baboon. Accordingly, our focus in primate research will be oriented toward this project. We believe that a success rate of as low as 50% will be a significant accomplishment and will allow us to more effectively examine the endocrine interrelationships between mother and fetus.

A considerable number of collaborative efforts are conducted between this branch and other research teams. In these instances we provide varying combinations of our surgical skills, our knowledge of perinatal medicine, and difficult to obtain fluid and tissue specimens. Among these projects are one in which we mince fresh fetal pancreatic islets and the investigators culture these in an attempt to grow large numbers of isolated beta cells. This growth has been successful on a short-term basis. The eventual hope is to grow enough cells to implant them into a glucose intolerant animal. Another project examines the roles of and the influencers of primate fetal liver glycogen synthetase and phosphorylase. This work has resulted in several publications which speak to net glycogen uptake and release by the newborn. We've completed a project in which we have looked at fovea development in fetal eyes and compared it to that which is known to exist in human. An extension of this will be the effects of light stimulation and the perception of light by the intrauterine fetus. We have contributed all of the gonads of our monkey fetuses to a group looking at testosterone metabolism in fetal life, and we have recently been asked to surgically approach the sheep and pig fetus in the first trimester in an attempt to utilize its immunologically immature system in the eventual therapy of human patients with metastatic cancer. We have utilized our monkey blood and amniotic fluid specimens to examine alpha feto-protein interrelationships between maternal and fetal circulations and amniotic fluid as well as amino acid profiles. We continue to collect lungs for enzyme analysis in the production of lecithin, and we have begun investigating the levels of zinc and copper in monkey and human amniotic fluid as a preliminary attempt to examine the metabolism of these trace metals in pregnancy.

The Perinatal Biochemistry Section seeks to define the mechanisms controlling production of steroid hormones during pregnancy and in early newborn life. Resolution of these problems requires manipulation of the mother, fetus and newborn, which are unacceptable in humans. However, we have shown that the baboon can be used as a model of pregnant and neonatal humans to pursue these problems. There is abundant evidence implicating steroid hormones in pregnancy maintenance, fetal maturation, and the onset of parturition. In addition, assays of steroid levels in maternal body fluids are frequently used clinically for monitoring feto-placental status in high-risk pregnancy. Appreciation of the factors normally regulating hormone production is essential to our understanding of the pathophysiology of the perinatal period. We are continuing to work with the baboon to provide the required information.

In the case of progesterone, we have established that the serum concentration metabolic clearance rate and blood production rate are increased in pregnant baboons. We have previously shown that corticosteroid binding globulin (CBD), which binds progesterone, is increased in pregnant baboons. It is probable that in pregnancy, changes in progesterone metabolism increase progesterone clearance and overcome the effects of increased binding to CBG. We are now determining the relative contributions of the ovary and placenta to the increased production rate. The factors which control utilization of circulating progesterone precursors by these organs are also being studied. Similarly, we have shown that the production of estrogens increases in pregnancy and that they are derived from circulating dehydroepiandrosterone (D) and its sulfate (DS). The concentrations of D and DS are 10-fold greater in newborn than maternal blood, suggesting that the fetus makes significant contributions of estrogen precursors. We are assessing the physiological significance of our finding that D is a more readily acceptable placental estrogen precursor than DS. It appears that over 80% of estradiol is derived from circulating D and about one-half of the estradiol formed from DS is converted *via* circulating D. This confirms that circulating D is quantitatively the major estradiol precursor. A reevaluation of the corresponding situation in pregnant women would be appropriate since in human pregnancy it is generally considered that DS is the major precursor and the importance of D has been largely overlooked. Cells of choriocarcinoma in culture have been used as a model to study regulation of estrogen production from neutral precursors. These cells form estradiol more readily from D than from DS and this appears to be due in part to selective protein binding of the conjugated precursor and to a relative sulfatase deficiency. Protein binding of DS may well contribute to differential utilization of D and DS *in vivo* but we have previously ruled out the possibility of a relative sulfatase deficiency in normal human term placenta. We have obtained evidence of differential cyclic AMP regulation of 3 enzymes of estrogen biosynthesis in these cultured cells and this too may contribute to preferential utilization of D over DS for estrogen production *in vivo*.

The adrenal production of estrogen precursors during pregnancy appears to be related to cortisol production by mother and fetus and we also have evidence of increasing fetal cortisol blood concentrations in late gestation. Changes in fetal metabolism of cortisol do not appear to be responsible for the increased cortisol levels. However, the mode of biosynthesis of cortisol does change with advancing gestational age. The combination of increasing weight and specific activity of the fetal adrenal 3β--hydroxysteroid dehydrogenase permits increased utilization of pregnenolone for cortisol production towards term. Utilization of progesterone or 11-deoxycortisol, although higher than that of pregnenolone at midgestation, does not increase in this way. These changes would be important for ensuring adrenocortical self-sufficiency of the neonate when placental progesterone is no longer available for cortisol production. Since cortisol competes with progesterone for binding sites on circulating CBG we are examining whether progesterone clearance may be influenced by variations in circulating cortisol.

The Pregnancy Research Branch also has as a mission the performance of clinical research projects. We have attracted both clinical obstetricians and pediatricians. Two clinical protocols were approved this year by the NIH Clinical Center Research Committee. One is entitled "The Effect of Vasodilator (Antihypertensive) Drugs on the Maintenance and Restoration of Fetal Well-Being in Patients with Hypertensive Disorders in Pregnancy." It is oriented toward defining in a specific way the effects of Apresoline on intrauterine fetal survival when the mother is hypertensive as adjudged by maternal estriol levels and the oxytocin challenge test. The other clinical protocol entitled "Response in Maternal Blood and Urinary Estrogens to a Bolus of Dehydroepiandrosterone (D) or its Sulfate (DS) in Normal Pregnancy" is an attempt to provide a accurate biochemical tool to assess antepartum fetal well-being. It has been recently decided that a clinical unit of the Pregnancy Research Branch (with specific commitment of beds and personnel) will not be initiated for the immediate future. This decision was based on considerations of patient safety, resources of the Clinical Center including personnel, and limitation of personnel in the branch. It does not negate the pursuit of these two clinical protocols via the use of adult beds on general medical or surgical services or in off-site areas. The proven nationally recognized strength of this branch is our significant contributions to the understanding of perinatal physiology and biochemistry through the animal models. The decisions to not dilute our resources with a clinical service will allow concerted effort without ambivalence toward proportioning of our resources of personnel, space, and budget as well as directed recruitment. We believe that our present activity and our intensified commitment to this basic research will result in a continuation of the growth and productivity of our group.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00106-05 PRB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Carbohydrate Metabolism during Primate Pregnancy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

Pregnancy Research Branch

SECTION

Perinatal Physiology Section

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.2	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

Accurately timed gestations in Rhesus monkeys are used. Streptozotocin, 40-48 mg/kg is administered intravenously to the adult female prior to or in the first trimester of pregnancy. This drug induces a glucose intolerant state secondary to pancreatic beta cell destruction. During the third trimester, acute experiments are performed under general anesthesia and aseptic surgical techniques. Cannulae are placed into both the maternal and fetal circulations to permit sequential sampling of blood. The responsiveness of the pancreatic cells to beta adrenergic agents as well as to somatostatin are examined by obtaining blood aliquots and analyzing them for glucose, insulin, growth hormone, and glucagon using biochemical and radioimmunoassay procedures. In some instances the same experiment in the newborn is performed on the first day of life after which tissue is examined histologically from both mother and conceptus. (Key words: carbohydrate metabolism, primate pregnancy).

Project Description:

Objectives:

1. To examine the responsiveness of the normal fetal pancreatic alpha and beta cells to the β-adrenergic agents which are being used as anti-labor drugs in humans.
2. To determine if the responsiveness of the fetal pancreatic beta cell in the glucose intolerant pregnancy is suppressed by somatostatin (somatotropin release-inhibiting factor, SRIF).
3. To determine amino acid concentrations in maternal and fetal serum and amniotic fluid as they relate to normal versus glucose intolerant pregnancies.

Methods Employed:

The pregnant rhesus monkey undergoes laparotomy under general anesthesia in the final 10% of gestation. A T-tube catheter is inserted into a fetal inter-placental vessel allowing administration of experimental agents and sampling of fetal blood while maintaining anatomic integrity of the amniotic cavity and feto-placental circulation. After a period of equilibration, one of the following two protocols is initiated.

1. One of three β-adrenergic agents is infused over 2, 10, or 30 minutes. Samples of maternal and fetal blood and amniotic fluid are obtained for at least 30 minutes following the infusion for determination of glucose, insulin, glucagon and growth hormone concentrations.
2. Glucose is administered twice as a bolus to each fetus with an interval of 30 minutes between boluses. A bolus of SRIF is administered in conjunction with one of the two glucose loads. Maternal, fetal, and amniotic fluid samples are obtained over 60 minutes following the initial glucose administration. Samples are analyzed for glucose, insulin, glucagon, and growth hormone.
3. Maternal blood, fetal blood, and amniotic fluid aliquots from both normal and glucose intolerant animals are obtained at laparotomy and submitted to amino acid analysis.

Major Findings:

1. β-adrenergic agents stimulate hepatic glycogenolysis, but do not stimulate secretion of either insulin or glucagon in the normal fetus. This is in contrast to evidence of both alpha and beta pancreatic cell stimulation by these agents in adults of various species, as well as in normal pregnant rhesus females in our colony. Further, in the fetus of a streptozotocin induced glucose intolerant pregnancy, these agents do stimulate insulin secretion.
2. Somatostatin does not appear to inhibit the fetal insulin response to hyperglycemia in the streptozotocin induced glucose intolerant pregnancy. This differs markedly from consistent inhibitions by SRIF of the insulin response to all variety of insulinogenic stimuli in adults of other species.
3. Preliminary amino acid analysis would indicate that fetal blood alanine levels may be elevated in the glucose intolerant pregnancy when compared to that of normal pregnancy. There seems to be no significant differences in maternal or fetal concentrations of other individual amino acids.

Significance to Biomedical Research and the Program of the Institute:
Evidence continues to accumulate that the pregnant Macaca mulatta provides a reproducible model of those findings that are now being delineated in carbohydrate metabolism of the pregnant human. The model continues to permit the examination of fetal responsiveness to various stimuli, an opportunity which is obviously not available in the human.

Proposed Course:
The development of a chronic primate model is projected for the current year. This will allow for the pursuit of lines of investigation which will largely parallel previous inquiries into carbohydrate metabolism but in a more physiologic, less acutely stressed anesthetized circumstance.

1. The responsiveness of the fetal endocrine pancreas to β-adrenergic stimulation will be further evaluated in glucose intolerant pregnancies on an acute basis. In normal pregnancies, the metabolic response to long term β-adrenergic therapy may be evaluated with maternal oral treatment utilizing the acute preparation and/or with prolonged maternal intravenous infusions in the chronic preparation.
2. The failure of somatostatin to inhibit fetal endocrine pancreatic secretion in the glucose intolerant pregnancy requires confirmation. Alternative dosage and modes of administration will be employed. Persistent lack of inhibition would represent an entirely unique pancreatic response to the agent and attempts will be made to elucidate the mechanism of escape.
3. Amino acid analysis will continue as acute preparations become available and will be extended to include sequential sampling in chronic preparations. Long term maternal infusions of insulinogenic amino acids in normal pregnancy will be contrasted to long term maternal glucose infusions in an effort to determine the factor(s) inducing fetal pancreatic beta cell responsiveness in the glucose intolerant pregnancy.

Publications:

Glinsmann, W.H., Eisen, H.J., Lynch, A. and Chez, R.A.: Glucose Regulation by Isolated Near-Term Fetal Monkey Liver. Pediat. Res. 9: 600-604, 1975.

Sparks, J.W., Glinsmann, W.H., Lynch, A. and Chez, R.A.: Glycogen Regulation in Isolated Perfused Near Term Monkey Liver. Pediat. Res. 10: 51-56, 1976.

Epstein, M.F., Farrell, P.M. and Chez, R.A.: Fetal Lung Lecithin Metabolism and the Amniotic Fluid L/S Ratio in Rhesus Monkey Gestations. Amer J. Ob/Gyn, 1975. (In Press).

Epstein, M.F., Farrell, P.M. and Chez, R.A.: Fetal Lung Lecithin Metabolism in the Glucose Intolerant Rhesus Monkey Pregnancy. Pediatrics, 1975. (In Press).

Epstein, M.F., Farrell, P.M. and Chez, R.A.: Lung Lecithin Synthesis in Primates as Related to Respiratory Distress Syndrome. In Advances in Medical Primatology, 1975. (In Press).

Farrell, P.M., Epstein, M.F., Fleischman, A.R., Oakes, G.K. and Chez, R.A.: Lung Lecithin Biosynthesis in the Nonhuman Primate Fetus, Determination of the Primary Pathway in vivo. Biol. of Neonate, 1975. (In Press).

Chez, R.A., Mintz, D.H. and Epstein, M.F.: Fetal Hormonal Mechanisms for Plasma Glucose Homeostasis in Normal and Glucose Intolerant Pregnancy. In *Early Diabetes in Early Life*. New York, Camerini-Davalas and Cole, Academic Press, 1975, pp. 141-163.

Epstein, M.F. and Chez, R.A.: Two Operative Techniques for Perinatal Research in the Rhesus Monkey. *Lab. Animal Sci.*, 1975. (In Press).

Chez, R.A.: Nonhuman Primate Models of Toxemia of Pregnancy. In Lindheimer, M.D., Katz, A.I. and Zuspan, F.P. (Eds.): *Hypertension in Pregnancy*, 1975. (In Press).

Epstein, M.F., Chez, R.A., Oakes, G.K. and Vaitukaitis, J.L.: Hypathalamic-Pituitary Control of Perinatal Prolactin Secretion in *Macaca mulatta*. *Endocrinology*, 1975. (In Press).

Walker, A.M., Oakes, G.K., Ehrenkranz, R.A., McLaughlin, M.K. and Chez, R.A.: Effects of Hypercapnia on Uterine and Umbilical Circulations in Conscious Pregnancy Sheep. *J. Appl. Physiol.*, 1975. (In Press).

Oakes, G.K., Chez, R.A. and Morelli, I.C.: Diet In Pregnancy. *Amer. J. Nursing*. 75: 1135-1136, 1975.

Chez, R.A., Haire, D., Quilligan, E.J., Wingate, M.B., Iffy, L. and Rakoff, A.: Status Controversies and Future of Obstetrical Care. *Fogarty Int'l Center Proceedings No. 22*, 1975 . (In Press).

Oakes, G.K. and Chez, R.A.: The Assessment of Fetal Well Being: An Overview. *Current Topics in Clinical Chemistry*. 1975. (In Press).

Chez, R.A., Burchell, R.C., Gray, M.J., Pion, R.J. and Settlage, D.F.: Sex in Pregnancy. *Contemp. Ob/Gyn*. 6: 99-128, 1975.

Chez, R.A. and Flood, B.: Record Keeping Aspects of Electrophysiological Fetal Monitoring. *Contemp. Ob/Gyn*. 6: 31-33, 1975.

Chez, R.A.: Clinical Applications of Nutrition in Perinatal Practice. In Moghissi, H. and Evans, T.N. (Eds.): *Harold C. Mack Symposium Nutrition & Human Reproduction: Biochemical & Clinical Aspects.*, 1975. (In Press).

Hobel, C.J., Aubry, R.H., Chez, R.A., Pernoll, M.L. and Schifrin, B.S.: Defining Normal Pregnancy. *Contemp. Ob/Gyn*. 6: 90-125, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00107-04 PRB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Estrogen Synthesis in Pregnant Baboons

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. D. Townsley	Chief, Perinatal Biochem. Sect.	PRB NICHD
OTHER:	H. A. J. Schut	Visiting Fellow	PRB NICHD
	S. E. Davis	Medical Officer	PRB NICHD

COOPERATING UNITS (if any)

Veterinary Medicine and Surgery Section, VR, DRS

LAB/BRANCH
Pregnancy Research Branch

SECTION
Perinatal Biochemistry Section

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	1.4	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to elucidate the mechanisms regulating estrogen production in primate pregnancy. The following approaches are being used: 1) identification and quantitation of the different circulating estrogen precursors; 2) determination of factors controlling availability of such precursors; 3) examination of the effects of variations in precursor availability on estrogen production; 4) determinations of placental biosynthetic capacity for estrogen synthesis. The studies also provide an evaluation of the baboon as a model of human pregnancy.

Project Description:

PROJECT NUMBER
Z01 HD 00107-04 PRB

Objectives:
To determine the relative importance of circulating precursor availability and placental biosynthetic capacity in regulating estrogen synthesis in vivo.

Methods Employed:
Dehydroepiandrosterone (D) and its sulfate (DS) serum concentrations were determined by a radioimmunoassay procedure adapted for use with baboons. Interconversions of D and DS and their relative contributions to circulating estradiol in baboons were determined by a double radioisotope constant infusion procedure.

Major Findings:
We have used two approaches to assess the physiological importance of our finding that both D and DS are utilized for estrogen synthesis in pregnancy but that maternal circulating D is a more readily acceptable estrogen precursor than DS. In pregnant and nonpregnant baboons concentrations of D and DS are significantly correlated with each other but DS levels are approximately 10-fold greater than those of D. However, mean maternal serum concentrations of both are similar in nonpregnant and pregnant animals. Concentrations of both steroids increase during gestation, so that by term levels of both are significantly greater than levels in nonpregnant animals. Serum D and DS concentrations are about 10-fold higher in the newborn (day 1) baboon than in the maternal circulation, a finding compatible with our previous suggestion that the fetus makes significant contributions of precursors for estrogen production in this species. The abundance of D and DS in both circulations points to placental factors, in addition to variations of precursor availability, in controlling estrogen production.

Initial experiments using continuous infusions of trace doses of D and DS to determine metabolic clearance rates and transfer constants, together with our estimates of D and DS mass, have clarified the interrelations between endogenous D and DS as estrogen precursors. Over 80% of estradiol is derived from circulating D, with less than one-fifth of this being converted to estradiol via DS. Conversely, less than 20% of estradiol is derived from circulating DS and of this amount one-half is converted via circulating D. Thus, quantitatively, circulating D is the major estradiol precursor. These results are in accord with our previous findings using exogenous loads of nonradioactive D and DS in pregnant baboons and determining relative conversions to urinary estrogens. If these findings are confirmed in subsequent experiments a reevaluation of the corresponding situation in human pregnancy will be appropriate. In pregnant women DS is generally considered to be quantitatively the major estradiol precursor and the importance of D has been largely overlooked.

Significance to Biomedical Research and the Program of the Institute:
Because of the widespread utilization of estrogen assays to monitor fetoplacental status and the apparent importance of such hormones in maintaining pregnancy and in the onset of parturition an understanding of the mechanism controlling their synthesis and metabolism is essential for definitive resolution of many problems of reproductive failure. Our approaches provide

fundamental knowledge of these processes which will ultimately be of use to the clinician. Elucidation of controlling mechanisms will require manipulative procedures in both mother and fetus, which are precluded in humans. An adequate animal model must be found to do this and our studies show that the baboon offers great promise for this purpose.

Proposed Course:
We will complete estimation of the extent to which estradiol is formed from DS *via* D as a circulating (as opposed to intraplacental) intermediate, from secreted D and directly from DS without circulating D as a discrete intermediate. The temporal relationships between circulating D, DS and estradiol in pregnant baboons will be determined using radioimmunoassay procedures. Better correlation between serum concentrations of D and estradiol than between DS and estradiol would provide additional evidence of the physiological importance of D as an estrogen precursor. Factors regulating production of D and DS by the fetal-newborn adrenal are incompletely understood. We will study the effects of trophic hormones on production of these steroids by newborn baboons *in vivo*.

Publications:
Townsley, J. D.: Utilization of dehydroepiandrosterone and its sulfate for estrogen production by pregnant baboons. *Acta Endocr*., 1976. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00111-04 PRB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Renin Angiotensin Relationships in Sheep Pregnancy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. A. Oakes	Medical Officer	PRB NICHD
OTHER:	K. S. Catt	Chief, Sec. on Hormonal Reg.	RRB NICHD
	R. A. Chez	Chief, Pregnancy Res. Br.	PRB NICHD
	S. C. Brennan	Guest Worker	PRB NICHD
	M. K. McLaughlin	Physiologist	PRB NICHD

COOPERATING UNITS (if any)

Office of the Director, National Heart & Lung Institute
Reproduction Research Branch, NICHD
Veterinary Resources Branch, Division of Research Services

LAB/BRANCH
Pregnancy Research Branch

SECTION
Perinatal Physiology Section

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.0	0.8

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this study are to study the effects of acute pertubations in maternal and fetal plasma electrolytes and volume on maternal and fetal plasma renin activity, and serum sodium and potassium and to study the effects of a low sodium diet on these same indices. Accurately timed gestations in Dorset sheep are utilized. Under anesthesia and aseptic surgical conditions, cannula are placed into the maternal and fetal circulations. The experiments are performed subsequently after the animal has recovered and is in a nonstressed nonanesthetized state. Measurements of renin activity are made utilizing radioimmunoassay techniques. (Additional key words: renin-angiotensin, sheep pregnancy).

PROJECT NUMBER

Project Description: Z01 HD 00111-04 PRB

Objectives:

1. To study the effects of acute perturbations of maternal and fetal plasma electrolytes and volume on maternal and fetal plasma renin activity, serum sodium and potassium.
2. To study the effects of a low sodium diet on maternal and fetal plasma renin activity, serum sodium, and potassium.

Methods Employed:

Accurately timed gestations in Dorset sheep are utilized. At 120-125 days of gestation (term 145 ± 5 d), catheters are placed into the maternal and fetal aorta and inferior vena cava. Experiments are performed 5-14 days postoperation. A sodium load, a volume load or a combined sodium volume load is given intravenously to mother or fetus. Arterial blood samples are obtained serially before, during, and after the infusion of the experimental solution. Similar experiments will be performed after the ewe has been on a low sodium diet for 14 days. Measurements of plasma renin activity, serum sodium and potassium are made on the obtained blood samples.

Major Findings:

These experiments are currently ongoing with the data being collected and analyzed. The results of the renin assays are not available at the time of this writing. Fetal survival after fetal intravascular catheterization in ewes on low sodium diets is markedly diminished compared to survival with normal sodium diets.

Significance to Biomedical Research and the Program of the Institute:

Factors and variables that regulate and influence the production and secretion of fetal renin in pregnancy are not known. The functional role of fetal renin is also not known. The fetal baseline levels of plasma renin actively are higher than the adult levels. Whether fetal renin is responsive to serum sodium and tonicity levels, like the adult, is unknown. The resulting vasoactive angiotensins could have important effects on fetal placental blood flow, blood volume, sodium metabolism and well being.

Proposed Course:

The principal investigator is leaving the Institute. Further investigation by others of the interrelationships of maternal and fetal plasma renin activity, angiotensin II, and aldosterone by interrupting the normal renin-angiotensin-aldosterone cycle with specific antagonists will be available.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00113-02 PRB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effect of Beta-Adrenergic Drugs on Placental Circulations of Conscious Pregnant Sheep

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. A. Ehrenkranz	Clinical Associate	PRB NICHD
OTHER:	A. M. Walker	Visiting Associate	PRB NICHD
	G. K. Oakes	Medical Officer	PRB NICHD
	L. A. Hamilton, Jr.	Medical Officer	PRB NICHD
	M. K. McLaughlin	Physiologist	PRB NICHD
	G. E. Sonberg	Biological Lab. Tech.	PRB NICHD
	S. R. Williamson	Biological Lab. Tech.	PRB NICHD
	R. A. Chez	Chief, Pregnancy Res. Br.	PRB NICHD

COOPERATING UNITS (if any)

Veterinary Resources Branch, Division of Research Services

LAB/BRANCH
Pregnancy Research Branch

SECTION
Perinatal Physiology Section

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.4	1.6

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this study are to examine the effect of certain B-adrenergic anti-labor drugs on uterine and umbilical blood flows, maternal and fetal heart rates, arterial pressures, arterial blood gases and plasma glucose concentrations, and to evaluate the effect of these agents on maintenance or restoration of fetal well-being. Pregnant Dorset sheep are operated between 125-145 days gestation (term = 145 days). Electromagnetic flow probes and catheters are placed on/in pertinent maternal and fetal vessels and chronic preparation is obtained. After a minimum of 48 hours to allow for recovery and reestablishment of maternal and fetal homeostasis, the test drug, ritodrine HCl, Salbutamol, or fenoterol (Th1165a), is infused into a peripheral maternal vein. It is administered at either a constant dose for 2 hours or a progressively increasing dose with step increments every 15 minutes. Physiologic measurements are then obtained utilizing the surgically placed flow probes and catheters. (Key words: placental circulation, sheep, pregnancy, anti-labor).

PROJECT NUMBER

Project Description: Z01 HD 00113-02 PRB

Objectives:

1. To examine the effect of certain β-adrenergic anti-labor drugs on uterine and umbilical blood flows, and maternal and fetal heart rates, arterial pressures, arterial blood gases, and plasma glucose concentrations.
2. To evaluate the effect of these agents on maintenance or restoration of fetal well-being.

Methods Employed:

Chronically instrumented pregnant Dorset sheep of 115-135 days gestation (term = 145 days) were used. Under ketamine infusion, electromagnetic flow transducers were applied to the main uterine artery of the pregnant horn and to the common intra-abdominal umbilical vein. Polyvinyl catheters were placed in a maternal and fetal aorta and inferior vena cava and in the amniotic cavity. After a minimum of 48 hours to allow for recovery and reestablishment of maternal and fetal homeostasis, the test drug (ritodrine, Th1165a, salbutamol, or isoxsuprine) was infused into the maternal or fetal inferior vena cava. The drugs were infused over 120 minutes as either a constant dose or a progressively increasing dose with step increments every 15 minutes. Measurements of uterine and umbilical blood flows, and maternal and fetal mean arterial pressures, heart rates, arterial blood gases, and plasma glucose concentrations were obtained utilizing the surgically placed flow probes and catheters.

Major Findings:

The data demonstrate that infusion of ritodrine, salbutamol, and isoxsuprine to the ewe result in significant decreases in uterine blood flow and mean maternal arterial pressure. Infusion of Th1165a to the ewe did not significantly alter these indices. Each agent produced a significant increase in maternal heart rate. During the final 30 to 60 minutes of the infusions of ritodrine and salbutamol to the ewe, uterine blood flow and maternal heart rate began to return to control levels, while mean maternal arterial pressure remained decreased. In a 120 minute postinfusion recovery period, following the infusions of Th1165a and salbutamol to the ewe, uterine blood flow increased significantly above control levels, as maternal heart rate and mean arterial pressure reached control values. During maternal infusions of each of these agents, umbilical blood flow and fetal heart rate either did not change or increased slightly; mean fetal arterial pressure remained relatively constant. Infusion of these agents to the fetus result in variable changes or increases in umbilical blood flow and fetal heart rate, and variable changes or slight decreases in mean fetal arterial pressure. Although maternal and fetal acid-base status remain within normal physiological limits, infusion of the drugs result in the development of a mild metabolic acidosis, with a slightly decreased pH and an increased base deficit in both the ewe and fetus. Maternal and fetal plasma glucose levels increase during the infusion.

Significance to Biomedical Research and the Program of the Institute:

The increased rate of neonatal morbidity and mortality associated with prematurity per se and fetal distress in labor have led to the current clinical interest in the use of antilabor drugs to inhibit uterine activity in the treatment of threatened premature labor, acute fetal distress in labor, and potentially ominous hyperdynamic labor. Clinical studies in Europe have demonstrated that infusion of β-adrenergic agents such as ritodrine, Th1165a, salbutamol and isoxsuprine to pregnant, laboring women arrest uterine contractile activity and result in the improvement of fetal acid-base status following acute intrapartum asphyxia. Our investigations are aimed at evaluating the maternal and fetal cardiovascular responses to these drugs in the absence of labor, so as to better understand the responses obtained during labor. Further, European physicians have been attempting to treat intrauterine growth retardation with these agents in the last third of gestation. The data has been contradictory.

Proposed Course:
The effects of these agents will be evaluated in similarly prepared sheep models during a prolonged infusion of greater than 8 hours. This will more accurately simulate therapy in the human, and will allow evaluation of the end-infusion and the post-infusion uterine blood flow increase, and investigation of possible accomodation by the utero-placental vasculature.

Publications:
Ehrenkranz, R.A., Walker, A.M., Oakes, G.K., McLaughlin, M.K., and Chez, R.A.: Effect of Ritodrine Infusion on Uterine and Umbilical Blood Flow in Pregnant Sheep. Am. J. Obstet. Gynecol., 1976. (In Press).

Walker, A.M., Oakes, G.K., Ehrenkranz, R.A., McLaughlin, M.K., and Chez, R.A.: Effects of Hypercapnia on Uterine and Umbilical Circulations in Conscious Pregnant Sheep. J. Applied Physiol., 1976. (In Press).

Oakes, G.K., Walker, A.M., Ehrenkranz, R.A., Cefalo, R.C., and Chez, R.A.: Uteroplacental Blood Flow During Hyperthermia With and Without Respiratory Alkalosis. J. Applied Physiol., 1976. (In Press).

Pepe, G.J., Ehrenkranz, R.A., and Townsley, J.D.: The Metabolic Clearance Rates and Interconversion of Cortisol and Cortisone in Pregnant and Nonpregnant Baboons. Endocrinology, 1976. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00114-03 PRB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cortisol Metabolism in the Baboon during Pregnancy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. J. Pepe	Senior Staff Fellow	PRB NICHD
OTHER:	J. D. Townsley	Chief, Perinatal Biochem. Sect.	PRB NICHD

COOPERATING UNITS (if any)

Veterinary Medicine and Surgery Section, VR, DRS

LAB/BRANCH

Pregnancy Research Branch

SECTION

Perinatal Biochemistry Section

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.1	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The project objective is to determine the mechanisms regulating fetal and maternal cortisol production in primate pregnancy. Current areas of interest, using the baboon as a model, include: 1) examination of fetal catabolism of cortisol and its potential role in attaining elevated serum cortisol at term; 2) determination of cortisol biosynthetic pathways in the fetal and newborn adrenal and the factors regulating their activities; 3) identification of urinary cortisol metabolites peculiar to pregnant and newborn baboons; 4) determination of the importance of protein binding and metabolism on the rate of clearance of cortisol.

PROJECT NUMBER

Project Description: Z01 HD 00114-03 PRB

Objectives:

1. To determine whether the increase in fetal serum cortisol (F) during gestation results from alterations in F metabolism.
2. To examine changes with gestational age in the capacity of the fetal adrenals to produce F.

Methods Employed:

Baboon neonates were obtained by spontaneous delivery at term or by cesarean section at various gestational ages. Changes in F metabolism were assessed by isolating metabolites from tissues 30 minutes after iv ^{14}C-F administration. Adrenal capacity to produce radiolabelled F was determined in homogenates using labeled substrates. In both types of experiments radiochemically pure metabolites were obtained by sequential chromatography and crystallization.

Major Findings:

Most ^{14}C from administered ^{14}C-F was found in liver; the intestine, brain and kidney accumulating substantial amounts. The majority ($> 75\%$) of ^{14}C in all tissues was unconjugated. In blood, F, cortisone and highly polar metabolites predominated. In liver, the major unconjugated metabolites were either reduced (e.g. tetrahydro-cortisol and -cortisone) or highly polar materials and only in liver were their concentrations greater than those in blood. In both liver and blood the F/cortisone ratio was > 2. In other tissues the predominant metabolite was cortisone, at a concentration exceeding that in blood. The metabolic pattern was similar in animals delivered prior to and at term. Only in animals delivered spontaneously and studied at 12 days of age were there significant changes. It is concluded that changes in F metabolism during intrauterine life cannot account for increasing circulating fetal F prior to term.

Production of F from pregnenolone was less than that from 11-deoxycortisol or progesterone but increased > 20-fold during the course of gestation, reaching a maximum at term. Utilization of 11-deoxycortisol or progesterone did not increase during the study period. When corrected for adrenal weight, which increased markedly during the study period, F production from pregnenolone increased > 80-fold during gestation, while that from other substrates remained constant. It is concluded that increased 3β-hydroxysteroid dehydrogenase-isomerase activity, combined with increased adrenal weight, permits increased utilization of pregnenolone for F synthesis towards term. These changes would be important for ensuring adrenocortical self-sufficiency of the neonate, when placental progesterone is no longer available for F production.

Significance to Biomedical Research and the Program of the Institute:

The importance of intrauterine developmental changes which prepare the fetus for adrenocortical self-sufficiency in an extrauterine environment are beginning to be appreciated. Fetal F may be involved in lung maturation and the onset of parturition as well as homeostatic maintenance during the perinatal period. The production of F during pregnancy is probably related to that of estrogen precursors; the importance of estrogens in pregnancy has been discussed elsewhere (see HD 00107-03). Elucidation of these processes is now being pursued using the nonhuman primate *Papio papio* and our studies have justified the use of this model.

Proposed Course:

Large amounts of progesterone and pregnenolone circulate in the developing fetus. The fetal adrenal has the capacity to utilize progesterone as early as mid-gestation but this ability remains constant thereafter. In contrast, the capacity to utilize pregnenolone *in vitro* increases with advancing gestation (see above). We will investigate factors regulating *in vivo* conversion of simultaneously infused, differentially labelled progesterone and pregnenolone to F by baboon neonates of differing gestational ages. The effects of trophic hormones on these conversions will be determined.

Much cortisone in the fetal circulation is of maternal origin and this could serve as a significant source of F if the 11β-hydroxysteroid dehydrogenase favors F production from cortisone rather than cortisone production from F. We will infuse differentially labeled F and cortisone into normal term and premature neonates to determine changes in F ↔ cortisone interconversion which may occur as gestation progresses.

Identification of two F metabolites isolated from newborn baboon urine (see HD 00114-02, 1975) will be completed. The potential interactions between F and progesterone in binding to maternal circulating corticosteroid binding globulin *in vivo* (see HD 00116-02, 1976) will continue to be investigated.

Publications:

Pepe, G. J., Ehrenkranz, R. A., and Townsley, J. D.: Metabolic clearance rates and interconversion of cortisol and cortisone in the baboon. *Endocrinology*, 1976. (In Press).

Pepe, G. J., and Townsley, J. D.: Cortisol metabolism by term baboon neonates. *Endocrinology*, 1976. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00115-03 PRB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Placental Mechanisms for Regulation of Steroid Synthesis in Primate Pregnancy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. A. J. Schut	Visiting Fellow	PRB NICHD
OTHER:	J. D. Townsley	Chief, Perinatal Biochem. Sect.	PRB NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pregnancy Research Branch

SECTION

Perinatal Biochemistry Section

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.6	PROFESSIONAL: 1.1	OTHER: 0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to identify mechanisms which regulate placental steroid hormone production. Two enzymatic mechanisms potentially regulating estrogen synthesis have been proposed as a result of our studies with disrupted preparations of human placenta. A choriocarcinoma cell line in culture is being used as an intact cellular model to evaluate these and other mechanisms regulating estrogen production. Aspects evaluated include: relative activities of biosynthetic enzymes metabolizing conjugated and unconjugated estrogen precursors; enzyme inhibition; differential protein binding of conjugated and unconjugated estrogen precursors; effects of permeability and of cyclic AMP.

PROJECT NUMBER
Z01 HD 00115-03 PRB

Project Description:

Objectives:
To identify the enzymatic mechanisms which potentially regulate placental estrogen synthesis.

Methods Employed:
The metabolism and extent of estrogen formation from neutral steroid substrates by intact and disrupted preparations of a cloned cell line of choriocarcinoma grown in culture (Flow Laboratories, Inc.) was determined by radioisotopic tracer methods.

Major Findings:
Preferential utilization of unconjugated dehydroepiandrosterone (D) over conjugated dehydroepiandrosterone sulfate (DS) for estradiol production by choriocarcinoma cells in culture has been confirmed. This is not due to differences in permeability to D and DS nor to sequestration of the steroid sulfatase. Selective binding of DS to serum albumin in the culture medium is partially responsible for this disparity but a deficiency of sulfatase activity, relative to subsequent enzymes of estrogen biosynthesis, also contributes to the difference in utilization of conjugated and unconjugated precursors. Estrogen formation from DS, D and androstenedione is increased by dibutyryl cyclic AMP and theophylline, indicating that sulfatase, steroid dehydrogenase and aromatase activities may be under cyclic AMP control. The choriocarcinoma sulfatase and steroid dehydrogenase are less susceptible to inhibition by steroids than corresponding enzymes of human term placenta. With the exception of the relative sulfatase deficiency and lack of susceptibility of these enzymes to inhibition, the choriocarcinoma in culture is a useful model of placental estrogen synthesis.

Significance to Biomedical Research and the Program of the Institute:
Estrogens appear to be important in pregnancy maintenance and possibly in the onset of labor. Assay of their levels in maternal body fluids are advocated for monitoring feto-placental studies. These studies add to our understanding of mechanisms regulating estrogen synthesis--an essential factor in resolving many problems of reproductive failure.

Proposed Course:
No further studies are planned at this time. The primary laboratory investigator (HAJS) will leave shortly and efforts are being concentrated on parallel investigations using the pregnant baboon as a model (see No. HD 00107-04).

Publications:
Townsley, J. D.: Inhibition of placental 3β-hydroxysteroid dehydrogenase by naturally occurring steroids. A potential mechanism regulating estrogen synthesis from unconjugated precursors. Acta Endocr. 79: 740-748, 1975.

ITHSONIAN SCIENCE INFORMATION EXCHANGE ROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00116-02 PRB

ERIOD COVERED

July 1, 1975 to June 30, 1976

TLE OF PROJECT (80 characters or less)

Progesterone in Primate Pregnancy

AMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. D. Albrecht	Staff Fellow	PRB NICHD
OTHER:	J. D. Townsley	Chief, Perinatal Biochem. Sect.	PRB NICHD

COOPERATING UNITS (if any)

Veterinary Medicine and Surgery Section, VR, DRS

BRANCH

Pregnancy Research Branch

TION

Perinatal Biochemistry Section

ITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

MANYEARS: 1.6	PROFESSIONAL: 1.1	OTHER: 0.5

Y OF WORK (200 words or less - underline keywords)

The general objective of this study is to elucidate the mechanisms regulating progesterone production in primate pregnancy. Using the pregnant baboon as a model the following aspects are under investigation: 1) determination of progesterone serum concentrations; 2) measurement of metabolic clearance rates and calculation of blood production rates of progesterone; 3) evaluation of the relative importance of serum protein binding and metabolism in determining clearance; 4) estimation of the contributions of placenta and ovary to total production; 5) examination of circulating precursors and factors controlling their conversion to progesterone.

HEW-6040

Project Description:

PROJECT NUMBER
Z01 HD 00116-02 PRB

Objectives:

1. To determine maternal serum progesterone (P_4) concentrations during normal baboon pregnancy.
2. To determine the metabolic clearance rate (MCR) and serum production rate of P_4 during baboon pregnancy.

Methods Employed:

Serum P_4 was determined by radioimmunoassay and P_4 MCR by a constant infusion of radiolabelled P_4.

Major Findings:

Serum P_4 concentrations were significantly greater in pregnant (12.0 ng/ml) than nonpregnant animals (4.9 ng/ml) during the luteal phase of the menstrual cycle. P_4 MCR was significantly greater in pregnant (1316 l/day) than in nonpregnant baboons (893 l/day). There was no correlation between P_4 MCR and gestational age. Serum P_4 production rates (MCR x serum concentration) were 4.6 mg/day in the luteal phase of the menstrual cycle and 15.3 mg/day during the last two-thirds of gestation. Thus increased serum P_4 concentrations in pregnant baboons result from increased production rather than decreased clearance. We have previously shown that corticosteroid binding globulin (CBG), which binds P_4 is increased in baboon pregnancy. It is probable that in pregnancy, changes in P_4 metabolism increase P_4 clearance and overcome the effect of increased binding to CBG.

Significance to Biomedical Research and the Program of the Institute:

P_4 is essential for maintenance of pregnancy and may be involved in the onset of parturition. However, there is a dearth of information on factors regulating its production in pregnancy. The increases in circulating P_4 concentrations and P_4 production rate in baboon gestation indicate that, in this respect, the baboon is superior to the rhesus monkey as a model of human pregnancy. Definition of mechanisms regulating P_4 production in this species will facilitate elucidation of the corresponding mechanisms in pregnant women.

Proposed Course:

Our preliminary observations in baboons suggest that, in addition to placental P_4 synthesis, a substantial portion of the P_4 produced during pregnancy is of ovarian origin. The relative importance of these sources will be assessed by determining P_4 concentrations in uterine and utero-ovarian veins of pregnant baboons. Ovarian-placental interrelations in P_4 production and the factors regulating utilization of circulating P_4 precursors will be investigated. Since cortisol competes with P_4 for binding sites on circulating corticosteroid binding globulin we will examine whether clearance of P_4 may be influenced by variations in circulating cortisol. Determinations of P_4 MCR prior to and after betamethasone suppression of cortisol production in pregnant animals and after exogenous cortisol administration will be made to explore this possibility. (See HD 00114-03 PRB).

Publications:

Albrecht, E. D., and Townsley, J. D.: Serum progesterone in the pregnant baboon (Papio papio). Biol. Reprod., 1976. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00117-01 PRB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effect of Alpha Agonist and Antagonist Infusions on the Sheep Placental Circulations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. K. Oakes	Medical Officer	PRB NICHD
OTHER:	R. A. Ehrenkranz	Clinical Associate	PRB NICHD
	A. M. Walker	Visiting Associate	PRB NICHD
	L. A. Hamilton, Jr.	Medical Officer	PRB NICHD
	S. C. Brennan	Guest Worker	PRB NICHD
	M. K. McLaughlin	Physiologist	PRB NICHD
	R. A. Chez	Chief, Pregnancy Res. Br.	PRB NICHD

COOPERATING UNITS (if any)

Office of the Director
National Heart and Lung Institute
Veterinary Resources Branch, Division of Research Services

LAB/BRANCH

Pregnancy Research Branch

SECTION

Perinatal Physiology Section

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 2.8	PROFESSIONAL: 2.0	OTHER: 0.8

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives are to examine the effects of the alpha agonist methoxamine and the alpha antagonist phenoxybenzamine on uterine and umbilical blood flows, maternal and fetal mean arterial pressures and heart rate as well as acid-base status. Accurately timed gestations in Dorset sheep are utilized. At 120-125 days of gestation, electromagnetic flow transducers are placed on pertinent fetal and maternal vessels, and catheters are placed onto the maternal and fetal aorta and inferior vena cava. Leads are exteriorized and the animal is allowed to recover. Experiments are performed at 3-13 days postoperatively. (Key words: alpha agonists, antagonists, sheep, placental circulations, pregnancy).

PHS-6040
(12-75)

Project Description:

PROJECT NUMBER
Z01 HD 00117-01 PRB

Objectives:
To examine the effects of the alpha agonist, methoxamine, and the alpha antagonist, phenoxybenzamine on uterine and umbilical blood flows, maternal and fetal mean arterial pressure and heart rate.

Methods Employed:
Accurately timed gestation in Dorset sheep are utilized. At 120-125 days of gestation (term 145 ± 5 d) electromagnetic flow transducers are placed on pertinent fetal and maternal vessels; catheters are placed into the maternal and fetal aorta and inferior vena cava. The leads are exteriorized. Experiments are performed at 3-13 days postoperation. Either methoxamine or phenoxybenzamine is infused intravenously for 60 min. into mother or fetus. The pertinent blood flows, mean arterial pressures, and heart rates are continuously recorded during a 30 min. control period, the 60 min. infusion period and for 240 min. post infusion. Fetal and maternal arterial blood samples are serially collected for analysis of pH, PCO_2 and PO_2.

Major Findings:
The intravenous infusion of phenoxybenzamine at this dosage into mother or fetus has not resulted in any significant changes in umbilical or uterine blood flow, or in maternal or fetal mean arterial pressure and heart rate. Methoxamine infusion produces significant increases in either mother or fetus mean arterial pressure associated with a bradycardia. The uterine blood flow decreases significantly with either maternal or fetal methoxamine. Umbilical blood flow does not change. In association with the decrease in uterine blood flow, the fetal arterial pH and PO_2 decrease and the PCO_2 increases.

Significance to Biomedical Research and the Program of the Institute:
The alpha adrenergic receptors have been shown to be present and responsive in both fetus and mother near term. Alpha adrenergic stimulation, whether through stress or pharmacologic utilization, can be potentially detrimental to the fetus.

Proposed Course:
None

Publications:
None

MITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00118-01 PRB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Effects of Propranolol Infusion on the Sheep Umbilical and Uterine Circulations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. K. Oakes	Medical Officer	PRB NICHD
OTHER:	A. M. Walker	Visiting Associate	PRB NICHD
	R. A. Ehrenkranz	Clinical Associate	PRB NICHD
	L. A. Hamilton, Jr.	Medical Officer	PRB NICHD
	S. C. Brennan	Guest Worker	PRB NICHD
	M. K. McLaughlin	Physiologist	PRB NICHD
	R. A. Chez	Chief, Pregnancy Res. Br.	PRB NICHD

COOPERATING UNITS (if any)
Office of the Director
National Heart and Lung Institute
Veterinary Resources Branch, Division of Research Services

LAB/BRANCH
Pregnancy Research Branch

SECTION
Perinatal Physiology Section

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this study are to examine the effect of propranolol infusions with its associated beta adrenergic blockade on uterine and umbilical blood flows, maternal and fetal mean arterial pressure and heart rate, gas and acid-base status. Pregnant Dorset sheep are operated between 120-130 days gestation. Electromagnetic flow probes and catheters are placed on and in pertinent maternal and fetal vessels allowing a chronic preparation to be obtained. After a minimum of 48 hours to allow for recovery and reestablish maternal and fetal homeostasis, the test drug is infused. (Key words: propranolol, sheep, pregnancy, placental circulation).

PHS-6040
(12-75)

Project Description:

Objectives:
To examine the effect of propranolol infusion with its associated beta adrenergic blockade on uterine and umbilical blood flows, maternal and fetal mean arterial pressure and heart rate, gas and acid-base status.

Methods Employed:
Accurately timed gestations in Dorset sheep are utilized. At 120-125 days of gestation (term 145 ± 5 d) electromagnetic flow transducers are placed on pertinent fetal and maternal vessels; catheters are placed into the maternal and fetal aorta and inferior vena cava. The leads are exteriorized. Experiments are performed at 7-13 days postoperation. Propranolol is infused intravenously for 60 min into mother (4 μg/kg/min) or fetus (10 μg/kg/min). The pertinent blood flows, mean arterial pressures and heart rates are continuously recorded during a 30 min control period, the 60 min infusion period and for 240 min post-infusion. Fetal and maternal arterial blood samples are serially collected for analysis of pH, PCO_2 and PO_2.

Major Findings:
Umbilical blood flow decreased 20% from control with both maternal and fetal intravenous propranolol infusion. There was no change in uterine blood flow or in maternal or fetal mean arterial pressure during either infusion. Maternal and fetal heart rate decreased 18% and 9% from control, respectively, during maternal infusion. Fetal heart rate decreased 15% from control during fetal propranolol infusion and maternal heart rate did not change. There was no change in maternal or fetal arterial pH, PCO_2 or PO_2 during either infusion.

Significance to Biomedical Research and the Program of the Institute:
Although the use of propranolol in human pregnancy has not been studied extensively there are individual case reports in which a mother taking propranolol during pregnancy has delivered a growth retarded infant who is asphyxiated at birth. The neonate's heart rate and the placental weight were both below normal levels. There could be a possible association between these clinical findings and the use of propranolol. This becomes more pertinent when it is appreciated that propranolol is increasingly being suggested for the therapy of preeclampsia and dysfunctional labor.

Proposed Course:
The decrease in umbilical blood flow could be due to an effect of propranolol itself or due to the associated beta blockade. Further examination of these two mechanisms will be considered.

Publications:
Farrell, P.M., Epstein, M.F., Fleischman, A.R., Oakes, G.K. and Chez, R.A.: Lung Lecithin Biosynthesis in the Nonhuman Primate Fetus: Determination of the Primary Pathway in vivo. Biol. Neonate, 1976. (In Press).

Walker, A.M., Oakes, G.K., Ehrenkranz, R.A., McLaughlin, M.K. and Chez, R.A.: The Effects of Hypercapnia on Uterine and Umbilical Circulations in Conscious Pregnant Sheep. J. Applied Physiology, 1976. (In Press).

Oakes, G.K., Walker, A.M., Ehrenkranz, R.A., Cefalo, R.C. and Chez, R.A.: Uteroplacental Blood Flow During Hyperthermia With and Without Respiratory Alkalosis. J. Applied Physiology, 1976. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00119-01 PRB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Circadian Rhythms in Placental Circulation of Conscious Near Term Pregnant Sheep

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. M. Walker	Visiting Associate	PRB NICHD
OTHER:	G. K. Oakes	Medical Officer	PRB NICHD
	R. A. Ehrenkranz	Clinical Associate	PRB NICHD
	M. K. McLaughlin	Physiologist	PRB NICHD
	D. W. Alling	Special Asst. for Biometry	OSD NIAID
	R. A. Chez	Chief, Pregnancy Res. Br.	PRB NICHD

COOPERATING UNITS (if any)

LAB/BRANCH

Pregnancy Research Branch

SECTION

Perinatal Physiology Section

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	1.2	0.7

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this study are a) to establish whether a circadian rhythm exists in uterine blood flow b) to establish whether a circadian rhythm exists in umbilical blood flow c) to examine the phase relationship between uterine blood flow and umbilical blood flow rhythms and d) to investigate the effect of environmental variables on the amplitude, phase and period of the rhythms. Chronically instrumented pregnant Dorset sheep of 129-131 days gestation are used. Under ketamine anesthesia electromagnetic flow transducers are applied to the dominant uterine artery and the common intra-abdominal umbilical vein. Catheters are placed in the maternal aorta, fetal aorta and amniotic cavity. These along with the transducer leads are exteriorized through a skin incision in the flank of the ewe. Experiments are performed 5 days postoperative to allow complete recovery from the surgical procedure, and after a minimum of 3-7 days preoperative acclimation. Environmental conditions are stringently controlled. (Additional key words: placental circulation).

Project Description:

PROJECT NUMBER
Z01 HD 00119-01 PRB

Objectives:

1. To establish whether a circadian rhythm exists in uterine blood flow.
2. To establish whether a circadian rhythm exists in umbilical blood flow.
3. To examine the phase relationship between uterine flow and umbilical flow rhythms.
4. To investigate the effect of environmental variables (light-dark, noise-silence, warmth-cold, activity schedules, chemical agents) on the amplitude, phase and period of the rhythms.

Methods Employed:

Chronically instrumented pregnant Dorset sheep of 129-131 days gestation (term 145 days) were used. Under ketamine anesthesia, electromagnetic flow transducers were applied to the dominant uterine artery and the common (intra-abdominal) umbilical vein. Catheters were placed in maternal aorta, fetal aorta and amniotic cavity. These, along with the transducers leads, were exteriorized through a skin incision in the flank of the ewe. Experiments were performed 5 days postoperation to allow complete recovery from the surgical procedure. Environmental conditions were as follows: room temperature 68-72°F; light-dark (LD) cycle of 12 hr, light from 0700-1900 hr; background noise 68-70 dB(A). Animals were caged, but not otherwise restrained, and allowed continuous access to food and water. Flow and pressure measurements were made at 15 min intervals over a period of 24 hr beginning at 0800 hr; arterial blood gases and pH were measured at 4 hr intervals. Hemodynamic measures were examined for rhythmic changes by harmonic analysis performed using a digital computer. A Fourier curve with first and second harmonic cycles of 24 hr and 12 hr period were fitted to each data series.

Major Findings:

Circadian (24 hour period) rhythms were found in uterine blood flow of all ewes. Smaller amplitude circadian rhythms were found in umbilical blood flow. In neither case were the rhythms synchronous with LD cycle, or between animals. However, a consistent anti-synchrony was found between the maternal and fetal rhythms within a given animal; the curves were close to 180 degrees out of phase. There were no consistent harmonic cycles in maternal arterial, fetal arterial, or amniotic fluid pressures.

Significance to Biomedical Research and Program of the Institute:

The pregnant sheep is a widely used animal model for study of utero-placental physiology. This study shows that rhythmic variations in utero-placental blood flow of this animal must be considered in experimental design and data evaluation. Recognition of the rhythms allows a more valid definition of normal levels of flow as a basis for physiologic, pharmacologic and pathologic studies. Previous study in monkeys has shown that uterine blood flow varies diurnally with concomitant variation in uterine activity. This present study established that uterine blood has a circadian rhythm in the absence of uterine activity. Circadian rhythms in umbilical blood flow, and a reciprocal relationship between uterine and umbilical flow, have not been previously described. These findings speak against the concept of the placenta as a widely dilated vascular bed showing no evidence of a fine physiologic control of its perfusion. Rather, interdependence of maternal and fetal flow in the utero-placental circuit, or dependence of either one on the other, needs to be considered. Circadian variability in the sensitivity of the utero-placental circulation to exogenously administered chemical agents needs consideration.

Proposed Course:
The principal investigator is leaving. Additional investigations could follow from the fact that this study has established that reciprocal circadian rhythms exist in uterine and umbilical flows of conscious, near-term sheep. This model could be used to pertubate either the maternal or fetal rhythm and examine the resultant phase relations to clarify the question of interdependence or dependence of maternal and fetal flow. The dislocation of the normal phase relationship with exogenous chemical agents and its subsequent effect on utero-placental blood flow could also be planned.

Publications:
Walker, A.M., Oakes, G.K., Ehrenkranz, R.A., McLaughlin, M.K. and Chez, R.A.: Effects of Hypercapnia on Uterine and Umbilical Circulations of Conscious Pregnant Sheep. J. Applied Physiol., 1976. (In Press).

Walker, A.M., Oakes, G.K., Ehrenkranz, R.A., McLaughlin, M.K., Chez, R.A. and Alling, D.W.: Twenty-Four Hour Rhythms in Uterine and Umbilical Blood Flows of Conscious Pregnant Sheep. Am. J. Obstet. Gynec., 1976. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00120-01 PRB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Chronic Restraint of the Near-Term Pregnant Baboon

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. A. Chez	Chief, Pregnancy Res. Br.	PRB NICHD
OTHER:	L. A. Hamilton, Jr.	Medical Officer	PRB NICHD
	S. C. Brennan	Guest Worker	PRB NICHD
	M. K. McLaughlin	Physiologist	PRB NICHD

COOPERATING UNITS (if any)

Veterinary Resources Branch, Division of Research Services

LAB/BRANCH

Pregnancy Research Branch

SECTION

Perinatal Physiology Section

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this study are a) to obtain a pregnant baboon preparation with fetal catheters and transducers in place which will survive in a nonlabor situation postoperatively for several days and b) to determine if prostaglandin inhibitors or beta-mimetic agents are necessary to obtain such a preparation. Pregnant baboons near-term will undergo laparotomy and transducers and catheters will be placed in the fetus. Use of labor inhibiting agents will be administered as necessary by the presence of uterine contractions. During recovery, the animal will be placed in a specifically designed restraining chair and vital signs will be monitored. When these objectives are obtained, it will then be possible to study the interdependence of fetal and maternal endocrine relationships without the adverse effects of acute stress secondary to anesthesia and surgery. (Key words: chronic restraint, pregnant, baboon).

PROJECT NUMBER
Project Description: Z01 HD 00120-01 PRB

Objectives:

1. To obtain a pregnant baboon preparation with fetal catheters and transducers in place which will survive in a nonlabor situation postoperatively for several days.
2. To determine if prostaglandin inhibitors or beta mimetic agents are necessary to obtain such a preparation.

Methods Employed:

A pregnant baboon will undergo laparotomy under general anesthesia in the final 10% of gestation. Polyvinyl catheters will be placed into the fetal left carotid artery and fetal left internal jugular vein. EKG leads will be attached to fetal skin. A catheter will be placed in the amniotic cavity and the incisions closed. Polyvinyl catheters will be placed into a maternal internal carotid artery and internal jugular vein. These catheters will be subcutaneously tunneled to exit from the top of the head. The fetal catheters will be brought out through a stab wound in the left side of the mother. At the initiation of anesthesia, a prostaglandin inhibitor, (fenoprofen, naproxen, or indomethacin) or a beta mimetic agent (ritodrine, salbutamol, or Th1165a) will be administered to the mother in a dose which is tritrated by the presence or absence of uterine contractions.

The recovery of the animal will be while placed in a restraining chair which consists of arm and head locks and no leg, hip, or abdominal constraints. Fetal and maternal vital signs as well as uterine activity will be monitored continuously on a recorder. When a nonactive uterine state is reached the anti-labor drug will be tapered. If the uterus remains quiescent, studies of fetal carbohydrate metabolism analogous to those previously emanating from this branch (See Project No. Z01 HD 00106-05 PRB) will be done.

Major Findings:

1. Three pregnant baboons have been restrained in this chair for lengths of 107, 109, 105 days. These animals were placed in the chair between the 65th and 75th day of gestation in contrast with those that were placed in the chair at approximately 140 days gestation who went on to labor and delivery within 7 days of restraint. The three animals that did not deliver did have chronic arterial catheterization in the carotid. In one of these three animals, fetal surgery was performed. Labor was not successfully inhibited by salbutamol with delivery occurring at 40 hours postoperative.
2. In the Macaca mulatta, reports indicate that indomethacin and fenoprofen have been effective in obtaining a chronic preparation.

Significance to Biomedical Research and the Program of the Institute:

The monkey and baboon anatomically, physiologically, and biochemically simulate those phenomena found in human pregnancy to a remarkable degree. Therefore, there is every evidence that they are important and valid models of human pregnancy. The difficulty with these animals continually has been that the uterus is irritable and can be stimulated by manipulation or incision.

This is in marked contrast to the condition found with both pregnant sheep and pregnant goats and is the reason why the latter have been extensively employed in fetal research. The majority of data that emanates from the non-human primate is obtained under either acute situations of surgery or immediate postoperative stress. It is extremely difficult to control the variables in a consistent way in this type of preparation. Therefore, to obtain a chronic model by initially using these drugs would be a very important and significant advance that would provide exciting research opportunities which are not available in the human.

Proposed Course:
The baboon is a relatively rare and exceptionally expensive research resource. Therefore, only a specific number of animals have been delineated for this project. The first two animals will be done after the time that this report is due. We will know within eight animals of the success of this approach, and there is no intention of prolonging the attempts past that number if not successful.

Publications:
None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00121-01 PRB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effects of Prostaglandins Upon the Placental Circulations in Pregnant Conscious Sheep

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. K. McLaughlin	Physiologist	PRB NICHD
OTHER:	A. M. Walker	Visiting Associate	PRB NICHD
	L. A. Hamilton, Jr.	Medical Officer	PRB NICHD
	S. C. Brennan	Guest Worker	PRB NICHD
	R. A. Chez	Chief, Pregnancy Res. Br.	PRB NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pregnancy Research Branch

SECTION

Perinatal Physiology Section

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this study are to characterize the uteroplacental circulating effects of exogenously administered prostaglandins in conscious pregnant sheep. Timed gestation Dorset sheep are utilized at 120-125 days gestation; electromagnetic flow transducers are placed on the common intra-abdominal umbilical vein and the main uterine artery of the pregnant horn. Venous and arterial catheters are placed in fetal and maternal circulations and all leads are exteriorized. At 72 hours postoperatively, prostaglandin $F_{2\alpha}$, prostaglandin E_2 or prostaglandin A_1 are administered to the fetus or mother. (Additional key words: placental circulations).

PHS-6040
(12-75)

Project Description:

PROJECT NUMBER
Z01 HD 00121-01 PRB

Objectives:
To characterize the uteroplacental circulatory effects of exogenously administered prostaglandins in conscious pregnant sheep.

Methods Employed:
Timed gestation Dorset sheep are utilized. At 120 to 125 days gestation (term 145 ± 5 days) electromagnetic flow transducers are placed on the common intra-abdominal umbilical vein and the main uterine artery of the pregnant horn. Venous and arterial catheters are placed in fetal and maternal circulations and all leads are exteriorized. At 72 hours postoperatively, prostaglandin $F_{2\alpha}$, E_2 or A_1 is administered to the fetus or mother in doses ranging from 0.5 to 100 µg/kg. Blood flow, pressure and heart rate responses are continuously recorded. Arterial blood samples are drawn from fetus and mother to assess the animal's condition.

Major Findings:
In very preliminary studies, PGE_2, administered via the fetal aorta, in doses ranging from 1 to 100 µg/kg caused a twenty to ninety percent reduction in fetal umbilical blood flow. Fetal blood pressure increased 32% at 1 µg/kg with a thirty percent decrease at 100 µg/kg. Fetal heart rate was unchanged at the lower doses with an eighty percent drop at 100 µg/kg. The uterine hemodynamics appeared unaltered by fetal administration.

$PGF_{2\alpha}$ administered via the fetal aorta in doses ranging from 0.5 to 100 µg/kg resulted in increases in fetal umbilical blood flow at 5 µg/kg of 14% with a maximum increase of 25% at 100 µg/kg. There was a 10% rise in fetal blood pressure after an initial drop of 10% at 50 to 100 µg/kg of $PGF_{2\alpha}$. Fetal heart rate decreased 10% at 5 µg/kg and 53% with 50 to 100 µg/kg doses. Uterine hemodynamics were unaffected by fetal administration. The effects of PGA_1 as well as the response to maternal administration of the prostaglandins have yet to be determined.

Significance to Biomedical Research and the Program of the Institute:
Prostaglandins are present in high concentrations in the uterus, placenta, amniotic fluid and fetal cord blood. They are released by a variety of physiological mechanisms including the events leading to parturition. This model allows investigation of the role of these substances in the regulation of utero-placental blood flow in pregnancy.

Proposed Course:
This project will be actively pursued in the coming fiscal year.

Publications:
None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00122-01 PRB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effect of a Prostaglandin Inhibitor, on the Placental Circulations of Conscious Pregnant Sheep

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. A. Hamilton, Jr.	Medical Officer	PRB NICHD
OTHER:	R. A. Chez	Chief, Pregnancy Res. Br.	PRB NICHD
	M. K. McLaughlin	Physiologist	PRB NICHD

COOPERATING UNITS (if any)

Veterinary Resources Branch, Division of Research Services

LAB/BRANCH

Pregnancy Research Branch

SECTION

Perinatal Physiology Section

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	0.3

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this study are to examine a) the metabolic disposition of naproxen in the latter third of gestation in the pregnant Dorset sheep, b) to examine the effect of the agent on uterine and umbilical blood flow, maternal and fetal heart rates, arterial and venous pressure, and arterial gases, and c) to evaluate the effect of the agent on maintenance or restoration of fetal well-being. Chronically instrumented pregnant Dorset sheep at 115-135 days gestation are used. Under ketamine infusion, electromagnetic flow transducers are applied to the main uterine artery of the pregnant horn and to the common intra-abdominal vein. Polyvinyl catheters are placed in the maternal aorta, fetal aorta, maternal inferior vena cava, fetal vena cava and in the amniotic cavity. After a minimum of 48 hours to allow for recovery and to reestablish maternal and fetal homeostasis, the test drug is infused into the maternal and fetal inferior vena cava using a range of doses. (Additional key words: placental circulation).

Project Description:

PROJECT NUMBER
Z01 HD 00122-01 PRB

Objectives:

1. To examine the metabolic disposition of naproxen in the latter third of gestation in the pregnant Dorset sheep.
2. To examine the effect of naproxen on uterine and umbilical blood flows, maternal and fetal heart rates, arterial pressures, and arterial blood gases, and acid-base.
3. To evaluate the effect of naproxen on maintenance or restoration of fetal well-being.

Methods Employed:

Chronically instrumented pregnant Dorset sheep of 115-135 days gestation (term = 145 days) will be used. Under ketamine infusion, electromagnetic flow transducers will be applied to the main uterine artery of the pregnant horn and to the common intra-abdominal umbilical vein. Polyvinyl catheters will be placed in the maternal aorta, fetal aorta, maternal inferior vena cava, fetal vena cava and in the amniotic cavity. After a minimum of 48 hours to allow for recovery and reestablishment of maternal and fetal homeostasis, the test drug will be infused into the maternal or fetal inferior vena cava, using three dose ranges yet to be determined. Naproxen half-life study in the sheep is currently being carried out in order to derive dose range guidelines. After a 30 minute control period, naproxen solutions will be infused at a rate of 2.0 ml/minute to the mother or 0.2 ml/minute to the fetus for a 120 minute test period, followed by a recovery period to last until flows return to baseline values. The drug will be infused either as a constant dose or a progressively increasing dose with step increments every 15 minutes. Measurements of uterine and umbilical blood flows, maternal and fetal mean arterial pressures, fetal venous pressure, heart rates, arterial blood gases and acid-base status will be obtained utilizing the surgically placed flow probes and catheters.

The specific test hypotheses inherent in this protocol are:

1. Naproxen administered to the mother will affect maternal hemodynamics and uteroplacental circulation as well as umbilical blood flow and fetal hemodynamics.
2. Naproxen administered to the fetus will affect fetal hemodynamics and umbilical blood flow as well as uteroplacental circulation.

Major Findings:

Naproxen disposition studies are presently being carried out, and when completed, we will proceed to Objectives 2 and 3.

Significance to Biomedical Research and the Program of the Institute:

In the U.S. 125,000 babies are born each year who are mentally retarded or physically handicapped by cerebral palsy. The majority of this neonatal morbidity (as well as mortality) is associated with prematurity per se. Fetal distress during labor is another significant factor contributing to the additional fetal and neonatal damage and death of premature as well as term babies. Because of these two unsolved and often unexpected dilemmas, current clinical interest has developed in the use of anti-labor drugs to inhibit uterine activity in the treatment of threatened premature labor, acute fetal distress in labor, and potentially ominous hyperdynamic labor. Clinical studies in Europe have demonstrated that infusion of beta-adrenergic agents such as ritodrine, salbutamol, Th1165a and isoxsuprine to pregnant laboring women often arrests uterine contractile activity and results in improvement of fetal acid-base status following acute intrapartum asphyxia. However, our findings with chronically instrumented sheep models in the latter part of gestation demonstrate that in the non-labor state, infusion of ritodrine, salbutamol and isoxsuprine results in significant decreases in uterine blood flow and mean maternal arterial pressure. Human studies with isoxsuprine during labor likewise indicate a drop in systolic pressure and dose-related maternal tachycardia and the subsequent development of a severe progressive fetal heart rate late deceleration pattern. This derangement of maternal-fetal homeostasis was later ameliorated with stoppage of the beta-agonist.

Since the above data mitigate against the clinical effectiveness of many beta-agonists in the control of premature labor, it is both timely and appropriate to seek other agents which may act more specifically on the uterus as "anti-labor" drugs, and yet are less likely to act on vascular smooth muscle. Clinical studies on the inhibition of human premature labor by the prostaglandin antagonist indomethacin showed uterine contractions to be arrested completely in 80% of the cases with excellent fetal salvage rate, and no significant change in maternal blood pressure, pulse or fetal heart rate during treatment. In vivo monkey studies with another prostaglandin antagonist also indicated an arrest of uterine contractility without significant maternal or fetal side effects. With this background of animal and human studies, it therefore appears that prostaglandin inhibitors hold greater therapeutic promise than the beta-mimetic agents for premature labor. The chronically instrumented pregnant sheep model will allow the evaluation of maternal and fetal cardiovascular responses to this drug in the absence of labor so as to better understand the responses obtained during labor.

Proposed Course:

This project is just beginning and will occupy a significant segment of next year's research activities. It is quite possible that as with indomethacin, naproxen can be used in acute dosage to maintain chronically the pregnant nonhuman primate in the latter third of gestation in a restraining chair with functioning fetal catheters and transducers. This exciting model would allow a more precise delineation of the endocrine, biochemical and physiological interrelationships between mother and fetus.

Publications:

Gottschalk, W. and Hamilton, L.A., Jr.: Regional Anesthesia II. Pudendal and paracervical blocks in Obstetrics. Local Infiltration for Cesarean Section. In Wynn, R.M. (Ed.): Obstetrics and Gynecology Annual. New York, Appleton-Century-Crofts, 1975, pp. 237-264

Gottschalk, W. and Hamilton, L.A., Jr.: Paracervical-Pudendal Blocks in Obstetrics. In Beard, R. (Ed.): Clinics in Obstetrics and Gynaecology. London, W.B. Saunders, 1975, pp. 565-577

Hamilton, L.A., Jr.: Clinical Simulations. In McGuire, C.H. and Solomon, L.M. (Eds.): Selected Problems in Patient Management. New York, Appleton-Century-Crofts, 2nd Edition, 1976. (In Press).

NICHD ANNUAL REPORT
July 1, 1975 through June 30, 1976
Laboratory of Molecular Genetics

The Laboratory of Molecular Genetics conducts research directed towards understanding the molecular processes involved in the transmission of genetic information and in its regulated expression during growth and differentiation. A variety of model systems are under investigation, ranging from bacterial and animal viruses to complex vertebrate regulatory responses involving the synthesis of specific antibodies and the development of specific organ systems. Taken together, the laboratory joins active workers in the areas of genetics, biochemistry, electron microscopy, immunology, virology and cellular biology to bring their skills to bear on problems of mutual interest. At present, the laboratory is comprised of nine research groups. Their activities are described in the phylogenetic order of the biological systems with which their research is primarily concerned.

A. Studies have been directed towards understanding the mechanism by which a virus, the bacteriophage λ, inserts its chromosome into the chromosome of its host and how this intracellular virus nucleic acid is incorporated into an infectious particle. Given the powerful genetic selection technique that has been described in previous annual reports and which was developed by this laboratory, a large number of mutants defective in their ability to integrate and excise viral genetic information have been obtained. These genetic lesions have been assessed by genetic and electron microscopic techniques and careful mapping studies have shown that three critical genes, the att, int and xis genes, involved in the attachment integration and excision of the virus geneome, are compactly located on a region of the viral chromosome not more than 1500 bases in length. The enzyme encoded by the int gene has been partially purified in collaborative studies and the product of int promoted recombination has been visualized. Curiously this takes the form of linked chains of DNA and its relevance to the process of recombination is being assessed.

In addition and in collaboration with other groups in this laboratory, the bacteriophage λ has been modified for use as a safer vector for the formation of recombinant DNA molecules. This phage should be of enormous value to a host of scientists whose research will now turn to the formation of recombinant DNA molecules utilizing DNA from prokaryotic organisms in combination with that of higher organisms.

Additional studies have been directed towards understanding the process of bacteriophage packaging and maturation. A detailed model has been devised which accounts for the fact that processing (cutting) of long λ genome precursors into λ size pieces is accompanied by their packaging into the phage head. (Dr. Robert Weisberg - Principal Investigator)

B. Studies have been directed towards understanding regulatory mechanisms which control an array of diverse biochemical and physiological activities in E. coli. These studies, as reported in previous annual reports, have been directed towards understanding one of the broadest control processes yet discovered in bacterial cells which exerts a pleiotropic regulatory effect upon the expression of approximately 50% of the bacterial genome. This regulation is governed by guanosine tetraphosphate (ppGpp), whose formation is determined by the _rel_ gene. These studies have led to the construction of transducing bacteriophage which will be useful in the elucidation of the mechanism of action and genetics of the _rel_ locus.

Other studies have resulted in the construction of bacterial episomes containing short regions of the ribosomal gene, encoding the ribosomal promoter (origin of transcription) site. These will be extremely useful in detailing the mechanism of action of quanosine tetraphosphate on gene expression in a purified system. In studies directed towards understanding the mechanism of action of this nucleotide, a protein has been isolated which is capable of binding to DNA only in the presence of guanosine tetraphosphate. (Dr. Michael Cashel - Principal Investigator)

C. Studies have been directed towards _understanding the sequences of reactions which are involved in the synthesis, processing and degradation of messenger RNA_. Specifically, the role of ribonuclease III, a ribonuclease which attacks double stranded RNA has been examined in detail. This enzyme, known to duplicate the specific processing steps required for the production of ribosomal RNA, has also been found to produce specific cleavages in RNA derived from adenovirus and vesicular stomatitis virus, as well as to produce 18S and 28S size RNAs from ribosomal RNA precursors derived from _Notophtalmus viridescens_.

Further progress has been made on the purification of ribonuclease III-type enzymés from higher organisms and two such activities have been isolated from chick embryo cells. (Dr. Robert Crouch - Principal Investigator)

D. Studies have been directed towards understanding _the assembly of RNA tumor viruses_ in a system which uncouples the synthesis of murine leukemia virus RNA from the terminal steps in viral maturation. It has been discovered that viral particles produced under these conditions lack the high molecular weight 70S RNA genome and appear to contain only 8S and 4S RNA species. These virions have been shown to be identical to genomic RNA-containing virions with respect to their structural proteins. This indicates that the viral genome is not required to direct the assembly of viral proteins into virus particles. It has also been found that the structural proteins are synthesized _de novo_ in the absence of viral RNA synthesis, leading to the conclusion that infected cells contain stable viral messenger RNA molecules. Since viral message is never detected in virions, it is also concluded that there are two intracellular populations of viral RNA molecules, one destined to serve as messenger RNA and the other to be encapsidated into extracellular particles. (Dr. Judith Levin - Principal Investigator)

E. Studies have been directed towards understanding _the mechanism by which the virus gains control of the eukaryotic host biosynthetic machinery and towards developing the electron microscope as a tool for use in macromolecular analyses_. This approach has made use of several diverse viral systems including polio- and adenovirus. Work with the poliovirus has led to the isolation and partial characterization of an intermeidate in the initiation stage of poliovirus mRNA translation. Inasmuch as polio is translated to the exclusion of host mRNAs, it is the hope that further studies of this intermediate will yield important information about virus usurpation of host biosynthetic properties. In addition, work characterizing the proteins involved in the transcription of the poliovirus has progressed; intermediates in the process of replication have been identified and sorted with respect to active and inactive complexes using radioautography.

The early proteins of adenoviruses have been further characterized and new protein elements identified. This virus, which causes tumors in hamsters and infects man, has been shown by antigenic analysis to be producing proteins in tumor-bearing hamsters. These proteins are also synthesized early in infected human cells. (Dr. Jacob V. Maizel - Principal Investigator)

F. The control of eukaryotic gene expression has been studied in cultures of human cells infected with human adenovirus. The time course of production of early viral proteins likely to represent control elements of gene expression has been measured in the infected cells. Furthermore, messenger RNA coding for individual viral proteins has been extracted from these cells and has been visualized in the electron microscope. A new EM technique has been employed in collaborative studies between this group and the Maizel group, to show that nuclei of infected cells contain the large RNA that comprises virtually the entire viral DNA, whereas the cytoplasmic messenger RNA classes are much shorter and hybridize to defined points of the viral genome, according to the map position of the encoded gene functions. (Dr. Heiner Westphal - Principal Investigator)

G. Studies have been directed towards understanding the processes involved in cellular differentiation using the embryonic chick lens as a model system. These studies which have quantitated the δ-crystallin mRNA during the process of lens cell differentiation from the very earliest phases of lens induction to terminally differentiating lens fiber cells, have shown that the δ-crystallin genes are not amplified during differentiation and have provided evidence that transcriptional activation is responsible for the expression of these genes. Additional studies have concerned the subunit composition and structure of embryonic δ-crystallin and have shown that it is comprised of four subunits with very similar, possibly identical, sequences of encoded amino acids. It has also been shown that δ-crystallin can be dissociated into dimers and renatured to a tetramer with a tertiary structure similar to that of the native protein. More recent studies have led to the partial purification of an mRNA corresponding to tubulin from embryonic chick brain. (Dr. Joram Piatigorsky - Principal Investigator)

H. Studies have been directed towards understanding the regulatory pathways effecting antibody synthesis at the cellular level. These have been directed towards delineating the role of (thymus-derived) T-cells of both the helper and suppressor types. A streptococcal exotoxin (SPE) has been shown to inhibit preferentially an activity thought to be controlled by suppressor T-cells. In fact, this observation lends strength to the notion that two populations of T-cells exist one of which has a helper function, the other a suppressor function. SPE appears to have no effect on the helper function. The mechanism of interaction of SPE with T-cells will be investigated during the next year. In addition, studies of nude (T-cell deficient) mice borne of homozygous nude mothers and reared by heterozygous mothers suggest that these animals may receive residual T-cell helper activity from heterozygous mothers. The question of whether this derives from a cell transfer or a factor transfer or some other mechanism will be investigated in the course of the next year. (Dr. Edgar Hanna - Principal Investigator)

I. Studies have been directed towards understanding the regulated expression and organization of two sets of mammalian genes, those corresponding to globin and those corresponding to immunoglobulin. These studies have been based upon much previous developmental work which resulted in a technique for the isolation and assay of specific genetic sequences. Most recently, the process of erythrodifferentiation has been analyzed in considerable detail using cultured erythroleukemic cells. While it was previously shown by this laboratory that globin mRNA appears first as a function of erythrodifferentiation, this sequence of events have been shown to be composed of the sequential expression of the α, and then the β globin genes. The sequence recapitulates oncological gene expression. Additional studies have identified a new reagent, butyric acid, which is able to induce erythrodifferentiation in cultured cells. With this reagent it could be demonstrated that gene replication, DNA synthesis and cell division are not required for the expression of the globin genes. This observation permits the formulation of much simplier models for the regulated expression of this gene group.

Further studies of the origin of immunoglobulin gene diversity strongly suggest that the variable region of the immunoglobulin mouse λ chain is represented but few, possibly one time in the mouse genome. This finding lends further support to the notion that immunoglobulin diversity arises through a process of somatic mutation in which specific regions of the immunoglobulin genome undergo highly specific genetic alterations. (Philip Leder - Principal Investigator)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00066-07 LMG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Control Mechanisms in Temperate Bacteriophage λ

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Weisberg	Microbiologist	LMG NICHD
OTHERS:	N. Sternberg	Senior Staff Fellow	LMG NICHD
	L. Enquist	Scientist	LMG NICHD
	A. Kikuchi	Visiting Associate	LMG NICHD

COOPERATING UNITS (if any)
Dr. H. Nash, Laboratory of Neurochemistry, NIMH; Dr. Martin Rosenberg, Laboratory of Molecular Biology; NCI; Dr. Kiyoshi Mizuuchi, Laboratory of Molecular Biology, NIAMDD; Dr. Clarence Fuerst, Department of Medical Genetics, Univ. of Toronto

LAB/BRANCH
Laboratory of Molecular Genetics

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 3	PROFESSIONAL: 3	OTHER: 0

SUMMARY OF WORK (200 words or less - underline keywords)

The long term goals of this project are to determine the mechanism of recombination of virus DNA with the host chromosome and to determine how intracellular virus DNA becomes infective (or matures). We have isolated and studied mutants of coliphage λ that are unable to perform these processes. An analysis of mutants defective in virus-host recombination has yielded an estimate of the number and sizes of the proteins required and has given information about how the proteins interact with the DNA. An analysis of mutants defective in virus DNA maturation has suggested a model for this process. The properties of both of these types of mutant have enabled us to develop a safer vector for the cloning of fragments of eukaryotic DNA. Electron microscopic observation of DNA that has undergone recombination in vitro indicates that the DNA must be in a covalently closed superhelical conformation before recombination can occur (in collaboration with Drs. K. Mizuuchi and H. Nash). We are currently attempting to determine the nucleotide sequences of the regions of the virus and host chromosomes that are involved in virus-host recombination.

PHS-6040
(12-75)

Project Description:

Objectives: To understand how a virus inserts its chromosome into the chromosome of its host; to understand how intracellular virus nucleic acid is incorporated into an infectious virus particle; to study regulation of virus gene expression.

Major Findings:

1. Previously we described a powerful genetic selection for excision defective λ mutants. We used it to isolate many deletion and point mutations in the integration-excision region of λ and to construct a detailed genetic map. We have determined the molecular sizes of the int and xis genes by measurements of heteroduplex DNAs with unpaired segments of known genetic length in the att-int-xis region. We have screened int and xis mutants for novel variants that may be impaired in interaction of int and xis proteins with each other or with att. We found no evidence of protein interaction between int and xis. One int mutant may be impaired in att recognition. No such variants were found in xis.

Two temperature sensitive int mutations were tested for their ability to promote integrative and excisive recombination at various temperatures. We found that excisive recombination promoted by temperature sensitive int protein is markedly temperature resistant while integrative recombination is extremely temperature sensitive. The former reaction may therefore require much less int product than the latter.

In collaboration with Dr. H. Nash, we found that partially purified int product from 2 int temperature sensitive mutants is temperature sensitive for integrative recombination in vitro.

2. In collaboration with Drs. H. Nash and K. Mizuuchi, we have examined with the electron microscope λ DNA that had been exposed to an extract containing int protein. The input DNA was in the form of covalently closed circles, each circle carrying two attachment sites. A high proportion of the input circles had undergone int-promoted recombination between the two attachment sites. We found that the recombinant molecules consisted of two circles that were connected to each other like adjacent links of a chain (catenated circles). We believe that catenation results from the presence of superhelical turns in the parental DNA at the time of recombination.

3. We have modified the λgt·λC vector made by R. Davis to make it more suitable for use as a cloning vehicle for recombinant DNA molecules We isolated a new amber mutation in the lysis gene S that allows the growth of large quantities of the virus in a small volume. we also introduced two amber mutations in the phage head assembly genes to reduce the probability of the phage reproducing in nature.

4. The DNA protein, product of λ gene D, is a major component of the phage head. It is incorporated into the outside of a precursor head that contains uncut DNA, and it is required for the cutting of that DNA to a monomer length.

We have found that λ deletion mutants that have lost 18% or more of their DNA can cut and package their DNA without D protein. This and other observations have led us to a model of how λ DNA enters, is fixed to, and is cut by the λ head. The critical assumptions of this model are that entrance of the DNA into the head alters the structure so that D protein can add and DNA cutting can occur, that the rate of DNA cutting is slow relative to the rate at which the head is filled, and that D protein is needed to stabilize the head after an amount of DNA equal to 82% of the monomer length has entered.

5. Gene D mutants of λ were used to select for deletions of other regions of the phage genome. When a λ Dam lysogen of a sup^- host is induced and the phage yield plated on a sup^- host, more than 25% of the plaques carry a deletion. This method for obtaining and quickly screening deletions is being used 1) to determine what host and phage functions affect deletion frequency and 2) to isolate partial deletions of the int-xis region and of other non-essential phage regions

6. It is possible to form infectious λ phage particles in vitro by mixing phage DNA with an extract from infected cells. We have found that the efficiency of this reaction depends on genome size: the DNA of deletion mutants is a poorer substrate than wild type DNA. Our evidence indicates that this size dependence reflects the efficiency of action of the phage FII protein. The known role of the FII protein is to join the phage head to the phage tail. We suggest that it has a second role: to bring the end of the DNA into the tail so that it can be injected into a cell after infection. If the genome size is smaller than that of wild type λ, the end of the DNA can retract into the head after cutting thus preventing FII protein from threading it into the tail.

7. We have shown that a λ mutant isolated by Dr. C. Fuerst defines a new λ gene, gene nul. The action of gene nul is required for maturation of λ DNA. Its location at the extreme left end of the λ chromosome suggests that it may be involved in cutting native λ DNA from a higher molecular weight precursor.

Significance to Biomedical Research and the Program of the Institute: The insertion and excision of viral (and other episomal) DNA is known to change the physiology of the host organism. In addition, these viruses (and other episomal elements) provide an important mechanism of genetic transfer and exchange in the microbial world. Viruses promote genetic transfer because of their ability to convert host genes to an infectious form by packaging them inside a shell of viral structural proteins. It is highly likely that such a successful symbiotic relationship has an analogy in the relationship between certain animal viruses and the cells of higher organisms. In fact it is known that animal viruses can package the DNA of their hosts although the biological significance of this phenomenon is unknown. Moreover, an understanding of the control mechanisms operative in these easily studied microorganisms has obvious relevance to an understanding of regulatory elements in higher organisms. The ability to direct the insertion of phage DNA to pre-determined locations on the host chromosome should prove to be an extremely useful and widely applicable method for isolating gene products and studying the control of bacterial gene expression.

Proposed Course of Project:

We plan to use temperature sensitive and suppressor sensitive int mutants to determine the level of int protein required for recombination between different attachment sites. We plan to isolate and characterize pseudorevertants of int and xis mutants in order to determine which other proteins, if any, interact with int and xis and whether int and xis interact with each other. We plan to search for host mutants that have a decreased or increased capacity to support site specific integration. We plan to isolate an RNA transcript that covers the attachment site and use it to determine the nucleotide sequence of the attachment site. We plan to examine catenated DNA molecules in order to determine the density and sign of the superhelicity of the parental DNA when it undergoes recombination.

Publications:

1. Sternberg, N. and Weisberg, R.: Packaging of prophage and host DNA by coliphage λ. Nature 256: 97-103, 1975.

2. Enquist, L. and Weisberg, R.: The red plaque test: A rapid method for identification of excision defective variants of bacteriophage lambda. Virology, in press.

3. Enquist, L., Tiemeier, D., Leder, P., Weisberg, R. and Sternberg, N.: Safer derivatives of bacteriophage λgt·λC for use in the cloning of recombinant DNA molecules. Nature 259: 596-598, 1976.

4. Fiandt, M., Gottesman, M., Shulman, M., Szybalski, E., Szybalski, W. and Weisberg, R.: Physical mapping of coliphage λ att^2. Virology, in press.

5. Sternberg, N.: A genetic analysis of bacteriophage λ head assembly. Virology, in press.

6. Sternberg, N.: Analysis of a rifampicin resistant mutation of Escherichia coli that interferes with the expression of late coliphage λ genes. Virology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00067-08 LMG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Integrative Control of Macromolecular Synthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael Cashel	Research Medical Officer	LMG NICHD
OTHERS:	Gad Glaser	Visiting Scientist	LMG NICHD
	Marcel Bouquet	Visiting Fellow	LMG NICHD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Genetics

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	2.4	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

This project is broadly concerned with how a cell can integrate different patterns of gene expression and coordinate diverse biochemical and physiological activities. Previous work has revealed the existence of two regulatory systems, each capable of broad pleiotropic effects. The regulatory system under study here is mediated by an unusual nucleotide (ppGpp) and is estimated to maximally control the expression of approximately half of the Escherichia coli enzymes. The second pleiotropic regulatory system is mediated by cyclic AMP and is thought to mediate regulation of about 5% of cellular proteins.

Present work concerns genetic definition of the bacterial chromosomal region encoding the enzyme responsible for ppGpp synthesis on the ribosomes in order to ask whether the synthesis of ppGpp is a cellular function essential to life and to construct mutants with excessively high and low levels of ppGpp for further studies. This year progress has been made to purify ribosomal genes in order to assess mechanisms of ppGpp regulation of genetic expression in a biochemically pure system at the transcription level.

Project Description:

Objectives: The aim of this project is to understand how cellular functions and activities are integrated to coordinate diverse patterns of gene expression. Such regulation is best displayed during cellular adaptation to change in nutritional environment. Here the regulatory system studied in detail is mediated by guanosine tetraphosphate (ppGpp) formed on ribosomes. The accumulation of ppGpp is provoked by amino acid deprivation, change in energy sources or anaerobiasis. Previous annual reports have considered specific positive and negative regulatory consequences of ppGpp accumulation on gene expression, intermediary metabolism, transport and cellular physiology.

Principal Findings:

1. Biosynthesis of ppGpp. No bacterial mutants yet exist completely lacking in ppGpp. Classically relaxed mutants do exist that have a specific defect in ppGpp accumulation during amino acid starvation but such mutants possess normal basal levels of ppGpp that can be elevated during certain conditions such as energy source starvation. Does this mean there are two enzymes capable of ppGpp synthesis and only one operates at a ribosomal site? Alternatively is there a single enzyme that can only be mutated to lose ribosomal function because absolute loss of activity is lethal? Two approaches have been taken, both predicated upon genetic analysis. The first is based upon attempts to obtain deletions arising in nearby genes. This analysis strengthens the notion that the *rel* A gene provides a function essential for cellular growth; no deletions were found. The second approach is to obtain amber mutations in the *rel* A gene that are conditionally suppressed by a temperature sensitive suppressor. We have constructed strains in which such mutants might be isolated and have begun screening for such mutants. In addition we have isolated and characterized a lambda phage insertion into a closely neighboring gene (*pyr* G). Transducing phage derivatives have enabled determining the fine structure of genes in this region. A direct consequence is the new ability to obtain cells with varying levels of ppGpp synthesizing enzymes for further regulation studies. Such cells can be derived either through lambda induction or through amplification of plasmid strains carrying the *rel* A gene.

2. Regulation of gene expression by ppGpp. Earlier reports have described promoter dependent ppGpp effects on gene expression in crude enzymatic systems. These effects are gene specific and are stimulatory in some cases, inhibitory in others. In order to understand how these regulatory events occur, we have rearranged existing genes carried by a lambda transducing phage into a plasmid so as to permit amplification of a small portion of early sequences of a ribosomal RNA gene. This isolate has been characterized by hybridization competition experiments, restriction enzyme digests and by electron microscopy. The existence of this plasmid provides a means of isolation of sizeable amounts of DNA bearing a ribosomal promoter for *in vitro* studies of ribosomal gene expression. RNA polymerase mutants which are apparently resistant to ppGpp effects can be compared to normal polymerase behavior to pinpoint the defect *in vitro*. Affinity column techniques will become possible for identifying promoter recognition elements.

As discussed in the preceding annual report, the presence or absence of large amounts of ppGpp apparently determines the activity of about half of the *E. coli* genes encoding protein structural information. Despite this enormous regulatory effect, no direct selection method for relaxed mutants is yet available. Accordingly we have constructed strain pairs of *E. coli* K12 derivatives resulting in steady state growth with high or low ppGpp levels in a stringent or relaxed genetic background respectively. We have found that normal strains are capable of growth on a large number of substrates whereas relaxed strains are specifically unable to grow. Isolation and characterization of revertants of relaxed strains, capable of growth on several otherwise unsupportive substrates, should provide a means of isolation of pleiotropic regulation mutants. In this manner we have isolated several mutants that have this phenotype but which have not changed in their ability to accumulate ppGpp. In a manner analogous to cyclic AMP pleiotropic regulation, such a system should facilitate understanding of the ppGpp pleiotropic effects on gene expression. Preliminary indications have been found of a protein that specifically binds to *E. coli* DNA cellulose columns in the presence of ppGpp, but not ATP and GTP.

Significance to Biomedical Research and the Program of the Institute: Integrative control processes in general and adaptive regulation to nutritional and environmental changes are common to all growing cells. The emerging pleiotropic regulatory systems show promise in understanding some features of cell development and many aspects of cell biology.

Proposed Course of Project: The genetic approaches described above will be pursued along with *in vitro* characterization of regulatory consequences of ribosomal gene amplification and *rel* A gene amplification.

Publications:

1. Cashel, M.: Regulation of bacterial ppGpp. *Annual Review of Microbiology* 29: 301-318, 1975.

2. Cashel, M.: Pleiotropic Regulatory Effects of ppGpp in Bacteria. In Copeland, J. (Ed.): *Regulatory Biology*. Ohio State University Press, 1975, in press.

3. Reiness, G., Yang, H-L., Zubay, G. and Cashel, M.: Effects of guanosine tetraphosphate on cell-free synthesis of *Escherichia coli* ribosomal RNA and other gene products. *Proc. Nat. Acad. Sci. USA* 72: 2881-2885, 1975.

4. Cashel, M., Hamel, E., Shapshak, P. and Bouquet, M.: Interactions of ppGpp structural analogs with RNA polymerase, *A. Benzon Symposium IX*, in press, 1975.

5. Hamel, E.: Activities of quanosine triphosphate analogues in reactions catalyzed by elongation factor Tu and initiation factor 2 of *Escherichia coli*. *Biochim. Biophys. Acta* 414: 326-340, 1975.

6. Hamel, E., Heimer, E.P. and Nussbaum, A.L.: Synthesis of deoxyguanosine polyphosphates and their interactions with the guanosine 5'-triphosphate requiring protein synthetic enzymes of *Escherichia coli*. *Biochemistry* 14: 5055-5060, 1975.

7. Cashel, M.: Regulation of ppGpp and pppGpp synthesis in bacteria. *PAABS Revista*, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00068-05 LMG

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Factors Influencing Genetic Transcription-Initiation and Termination

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert J. Crouch	Research Chemist	LMG NICHD
OTHERS:	Stanton H. Hall	Research Associate	LMG NICHD
	Marie-Luise Dirksen	Guest Worker	LMG NICHD

COOPERATING UNITS (if any)

Dr. Robert Lazzarini, Laboratory of Molecular Biology, NINCDS
Dr. David Reese, Lung Cancer Branch, NCI; Dr. Bernard Moss, Laboratory of Biology of Viruses, NIAID; Dr. C.H. Wei, LBV, NIAID

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

SUMMARY OF WORK (200 words or less - underline keywords)

RNA plays an important role in cellular regulation - either by its presence in active form or by its total absence. A number of proteins have been shown to be involved in regulating RNA synthesis and RNA processing in *Escherichia coli* - among these are RNA polymerase, rho and RNase III. These proteins are involved in the synthesis or inhibition of synthesis of specific species of RNA and in the processing of RNA. Another enzyme which may be involved in regulation of RNA synthesis is RNase H. The objective of this research project is to determine the method by which several of these enzymes act to regulate RNA synthesis in *E. coli* and chick embryos.

PHS-6040

Major Findings:

Most of the effort of this laboratory, during the fiscal year 1976, has revolved around the study of enzymes that have been demonstrated to be involved in RNA processing (i.e., conversion of an RNA precursor to the form observed in most normal cells) or enzymes which have certain similarities to the processing enzyme. The prototype enzyme being employed is E. coli RNase III. Previously we have shown that most RNAs, which we have examined, are resistant to cleavage by this enzyme unless sufficiently high ratios of enzyme to RNA are examined. Even at these elevated enzyme concentrations specific cleavages of adenovirus messenger RNA and 18S and 28S ribosomal RNA of KB cell were observed. Basically, we concluded that sites, which are normally recognized as processing sites by this bacterial nuclease, are absent from messenger RNA of adenovirus and mature ribosomal RNA of KB cells. However, the specificity of the cleavages of these RNAs under high enzyme concentrations suggests that RNase III might be a very useful tool for studying various aspects of RNA composition.

Experiments have begun in collaboration with Dr. Robert Lazzarini utilizing RNase III and RNA from Vesicular Stomatis Virus (VSV). Dr. Lazzarini has been studying the structure of the RNA found in VSV and defective particles of VSV, and he has shown a variation in the amount of RNA from a variety of these defective particles. Fragmentation of VSV RNA and RNA from the defective particles could clearly be useful in understanding the basis for the differences in the RNAs as well as for cutting the more complex VSV RNA to fragments that are more manageable than the intact viral RNA for sequence determinations. We have achieved specific fragmentation of VSV RNA and are looking forward to studying differences amongst the RNAs from the defective particles.

A second attempt to probe eukaryotic RNAs with RNase III demonstrated the randomness of sites cleaved by RNase III with respect to the 5' termini of a general population of messenger RNAs from HeLa cells. Previously, we showed that 28S RNA of KB cells has a number of points at which RNase III can attack but one site is more susceptible than all of the others. We also demonstrated a hierarchy of sites in comparing one adenovirus messenger RNA with another. Most messenger RNAs from eukaryotes have some features in common (e.g., polyadenylate sequences at the 3' terminus and a 7-methylguanosine moiety at the 5' terminus). In experiments carried out in collaboration with Drs. Moss and Wei, we asked if there is a site near the 5' termini of HeLa cell messenger RNA that is common to all mRNAs. The results obtained showed that no special cleavage point exists adjacent to the 7-methylguanosine and that sites for RNase III attack near the 5' terminal portion are no more or less prevalent than for the internal regions of the messenger RNA molecules.

Both of the experiments described above depend on cleavage by RNase III using extraordinarily high ratios of enzyme to substrate. If we examine these same RNAs under conditions in which RNase III demonstrates a more stringent requirement for double-stranded RNA or sites which RNase III has strong interactions, no messenger RNA degradations are observed. Examination of ribosomal RNA processing in E. coli and in higher organisms encourages us to believe that enzymes such as RNase III - and sites in the ribosomal RNA precursor molecules - might be present in eukaryotic organisms. Careful treatment of ribosomal RNA precursors from Notophthalmus viridescens, a newt, with RNase III does, indeed, generate 18S and 28S RNA. This latter finding has a great deal of significance since it suggests that the mechanism of processing the ribosomal RNA precursor has remained stable during evolution (i.e., an enzyme from eukaryotes recognizes a sequence similar to that which the bacterial enzyme recognizes).

Such observations have led us to the second category of major findings. We have searched for enzymes in eukaryotes that are analogous to RNase III of E. coli with the hope that they will have a role in processing ribosomal RNA and/or messenger RNA. From 9 day chick embryos we have partially purified and characterized two enzymes that solubilize double-stranded RNA. One of these enzymes is found in the nucleus and one in the cytoplasm. They can be distinguished on their chromatographic behavior, their divalent metal ion requirements and their specificity toward poly rA.U and poly C.poly G. Both enzyme fractions still are contaminated with other nucleases (or are not specific for double-stranded RNA) and thus are not yet useful for studying specific processing of ribosomal RNA precursors.

In the course of purification of one of these enzymes we have observed another nuclease that has a specificity for single-stranded RNA and acts in an exonucleolytic fashion from the 3' terminus and progresses toward the 5' termini generating 5' mononucleotides. The 5' terminus of the original RNA remains as a dinucleotide.

Proposal for future work:

Examination of RNase III and probing eukaryotic RNAs with RNase III

A). An examination of VSV RNA and RNA of defective particles will be an informative study for analysis of RNA with RNase III and an important contribution to understanding VSV.

B). How much RNase III is still present in RNase III mutant? The necessity of RNase III as a cellular protein remains unclear. The level of RNase III which has been set by enzymatic assay is less than 1% of the normal value. By producing antibody to RNase III it should be possible to extend the limits of detection substantially.

C). With antibody produced against RNase III, it should be possible to visualize RNase III binding sites in the electron microscope. Since RNase III binds specifically in some RNAs such as T7 as well as to any double-stranded RNA, visualization of both types of sequences are envisioned.

Chick Embryo Enzymes

Further purification as well as tests with T7 early precursor RNA, ribosomal precursor from E. coli, and ribosomal precursor from Notophthalmus viridescens will be performed to establish any relationship of these enzymes to RNA processing.

Publications:

1. Crouch, R.J.: An Examination of Immune RNA. In Fink, M. (Ed.): Immune RNA in Neoplasia, New York, Academic Press, 1976, in press.

2. Crouch, R.J.: Ribonucleases and Factors Influencing Their Activities. In Fink, M. (Ed.): Immune RNA in Neoplasia, New York, Academic Press, 1976, in press.

3. Crouch, R.J.: Summary of Workshop Session on Immune RNA. In Fink, M. (Ed.): Immune RNA in Neoplasia, New York, Academic Press, 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00069-04 LMG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Molecular Aspects of the Replication of Enveloped Animal RNA Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Judith G. Levin	Research Biochemist	LMG NICHD
OTHERS:	None		

COOPERATING UNITS (if any)

Brenda Gerwin, Research Biochemist, Laboratory of Tumor Virus Genetics, NCI;
Wallace Rowe, Chief, Laboratory of Viral Diseases, NIAID

LAB/BRANCH
Laboratory of Molecular Genetics

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.3	PROFESSIONAL: 1.0	OTHER: 0.3

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to define the molecular mechanisms involved in the replication of enveloped RNA viruses and in particular, to understand factors which influence the regulation and expression of viral genetic information. The possible role of the viral RNA genome in directing virus assembly is currently being investigated in a system which uncouples murine leukemia virus RNA synthesis from terminal steps in virus maturation. The virus particles produced under these conditions resemble deletion mutants since they lack the 70S RNA genome and appear to contain only low molecular weight RNA species. In view of our recent results demonstrating that the defective virions contain all of the standard virus structural proteins, it will be of interest to further characterize virion RNA and to determine whether this RNA is derived from the host or whether a portion is virus-specific. In addition, studies on the properties of the virion-associated reverse transcriptase enzyme will be continued.

Project Description:

Objectives: To study factors which influence expression of viral genetic information; to define in molecular terms the specificity requirements of the virus assembly process; to understand the virus-host relationship in chronic infection.

Methods Employed: Mouse cells chronically infected with murine leukemia virus are maintained as continuous lines in culture. Leukemia virus production is measured by assay of the extracellular fluids for reverse transcriptase activity in response to synthetic templates. Infectivity is determined by the XC plaque assay. The RNA species present in virions are analyzed by polyacrylamide gel electrophoresis. Conditions have been devised which yield high resolution of a wide range of RNA molecules, from the high molecular weight 70S viral RNA genome to the low molecular weight 8S and 4S RNA species. Viral proteins are analyzed on polyacrylamide slab gels. Electron microscopy is used to follow viral morphogenesis and to delineate the ultrastructural features of the virions.

Major Findings:
1. In earlier work on this project, a system was developed in which biosynthesis of murine leukemia virus RNA is uncoupled from terminal steps in virus maturation by treating chronically infected cells with actinomycin D. Despite the absence of ongoing viral RNA synthesis, virus particles continue to be assembled for at least 8-12 hours. Most of the virions produced are defective, however, and form a unique and previously undescribed class of RNA tumor virus particles which lack 70S genomic RNA and appear to contain only low molecular weight RNA species.
2. The defective particles exhibit high levels of reverse transcriptase activity in response to exogenous primer/templates, indicating that the enzyme can be packaged within virions in the absence of its natural 70S RNA template. The enzyme associated with the defective particles has been purified in parallel with enzyme from standard virions on a larger scale than was possible previously. Studies carried out with the two preparations have thus far revealed very similar biophysical and catalytic properties.
3. Previous experiments with cycloheximide have shown that assembly of murine leukemia virus particles is dependent upon protein synthesis. Not surprisingly, we now find that viral protein synthesis accompanies virus assembly in actinomycin D-treated cells.

a. When cells are labeled with radioactive amino acid in the presence of actinomycin D, the amount of radioactivity incorporated into virions parallels the total amount of virion-associated reverse transcriptase activity (approximately 40% of control levels). Analysis of the labeled proteins by polyacrylamide gel electrophoresis reveals that although virions produced in actinomycin D-treated cells are defective with respect to their RNA content, they contain all of the standard virus structural proteins and in the correct proportions.

b. The results indicate that actinomycin D-treated cells contain stable viral messenger RNA molecules which continue to function for many hours in the absence of viral RNA synthesis. Since viral messenger RNA is not detected in virions even when the supply of viral RNA is severely limited, it is concluded that murine leukemia virus-infected cells contain two nonequilibrating pools of viral RNA molecules, one associated with polyribosomes and one which is encapsidated into extracellular particles. The two pools are defined experimentally by the difference in the half-lives of viral messenger RNA and virion or genomic RNA, i.e., 6-8 hours and 1.5-2 hours, respectively.

c. The results also demonstrate that the presence of the viral RNA genome, at least in its usual high molecular weight form, is not required for assembly of viral proteins into virus particles.

Significance to Biomedical Research and the Program of the Institute: The advantages of viral systems as models for studying the regulation and expression of genetic information are well recognized. Studies on the RNA tumor viruses are of special interest since these viruses establish a chronic infection in the host cell by integrating viral genetic information into the host chromosome. An understanding of the various steps involved in tumor virus replication and mechanisms for generating defective virions should aid in efforts to limit the oncogenic potential of these agents. Finally, information on the degree to which host material can be assembled into virus particles could have some application in the field of gene therapy, where the use of pseudotype virions has been contemplated as a means of introducing "foreign" nucleic acid into an individual possessing an inherited biochemical defect.

Proposed Course of Project: In the coming year, efforts will continue to obtain a more detailed knowledge of the biochemical and structural properties of the defective murine leukemia virus particles generated in the presence of actinomycin D. The low molecular weight RNA species associated with these particles will be studied with a view towards determining (a) the subviral location of the RNA; (b) whether the RNA is of host and/or viral origin; and (c) whether the reverse transcriptase tRNA primer is among the 4S RNA species encapsidated in the particles. Studies on the catalytic properties of the virion-associated reverse transcriptase will continue and with the development of methods to obtain larger amounts of purified enzyme, it should be possible to investigate the ability of the enzyme to copy natural RNA's. Electron microscopic studies of defective virions and core particles are also being contemplated.

Publications:

1. Levin, J.G. and Rosenak, M.J.: Synthesis of murine leukemia virus proteins associated with virions assembled in actinomycin D-treated cells: evidence for persistence of viral messenger RNA. Proc. Nat. Acad. Sci. USA (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00070-16 LMG

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Morphogenesis of Animal Viruses During Infection of Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jacob V. Maizel, Jr.	Research Chemist	LMG NICHD
	Margaret Stewart	Visiting Associate	LMG NICHD
OTHERS:	Jurg Meyer	Guest Worker	LMG NICHD
	Ronald Lundquist	Staff Fellow	LMG NICHD
	William Chin	Research Associate	LMG NICHD
	Masahidi Ishibashi	Guest Worker	LMG NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	5	0

SUMMARY OF WORK (200 words or less - underline keywords)

Using mammalian virus systems and combined electron microscopic and biochemical methods, in vivo intermediates in poliovirus translation have been detected and isolated, in vitro poliovirus RNA replication intermediates have been isolated and characterized, new early proteins of adenovirus have been localized, and high resolution molecular mapping of viral genomes has begun.

PHS-6040
(12-75)

Project Description:

Objectives: To identify the macromolecules produced by virus infection of human cells and describe their spatial and functional roles in replication of the virus and regulation of the host cell.

Methods Employed: Techniques for sensitive biochemical and biophysical characterization of proteins and nucleic acids are central to these studies. Electrophoretic methods have been developed permitting separation of 50-100 different sizes of protein or nucleic acid molecules and estimation of their amounts, sizes and number. Electron microscopic techniques are employed to characterize the 3-dimensional organization of proteins and nucleic acids in viruses and cell organelles, and a primary objective is to extend the usefulness of electron microscopy for macromolecular characterization of proteins and nucleic acids. Viruses representing widely different groups are used in conjunction with continuous mammalian cell culture to produce simplified and manageable mammalian systems for molecular biological studies.

Major Findings: Work has been concentrated in two groups of human viruses: picornavirus (RNA genome of 2.6×10^6 daltons) and adenovirus (DNA genome of 23×10^6 daltons).

Poliovirus has been studied with a view to understanding the unique features by which this virus nucleic acid is used as a message for translation and a genome for replication. Pactamycin, a drug that selectively inhibits protein translation at the initiation stage, has been used to isolate from infected cells 100S complexes containing newly made RNA, viral protein and cellular protein. These structures have been formed to originate from complexes larger than 400S in the infected cells. New structures with the properties of intermediates in both translation and RNA replication have also been observed.

Electron microscopic radioautography of viral RNA synthesis in highly purified enzyme-templates complexes using radioautography has allowed identification of active and inactive molecules. The active molecules resemble in structure the isolated "replicative intermediate" form of RNA. Enzyme molecules have not yet been seen in electron micrographs.

Adenovirus early proteins have been further characterized and additional proteins observed. New proteins of Mw 42K and 18K have been found. The 42K protein found in vitro confirms the detection of a 42K in vitro synthesized protein from other laboratories, which has been implicated in oncogenic cell transformation. E2a and b have been localized in the plasma membrane and E3 has been located in the inner nuclear membrane fraction. Antigenic analysis has shown specific reaction of serum from tumor bearing hamsters with early proteins of infected human cells.

Proposed Course of Project: The work will continue using biochemical fractionation in conjunction with electron microscopy to study the structure of viral replicating intermediates and to relate these to their functions. In the coming year effort will resume on oncornaviruses whose genomes are now better understood. Further molecular electron microscopic techniques will be the analysis of the genetic content of a number of additional viruses.

Publications:

1. Chin, W. and Maizel, J.V.: The Polypeptides of Adenovirus. VII. Further Studies of Early Polypeptides In Vivo and Localization of E2 and E2A to the Cell Plasma Membranes. Virology (in press).

Proposed Course of Project: The work will continue using biochemical fractionation in conjunction with electron microscopy to study the structure of viral replicating intermediates and to relate these to their functions. In the coming year effort will resume on oncornaviruses whose genomes are now better understood. Further molecular electron microscopic techniques will be the analysis of the genetic content of a number of additional viruses.

Significance to Biomedical Research and the Program of the Institute: Biochemical characterization of the early events in viral infections is more completely defining critical events in cellular and viral regulatory mechanisms. New techniques using electron microscopy are now permitting visualization of molecules in the process of carrying out their regulatory roles. Observation of the specific interactions between cellular and viral genomes and their transcriptional and translational products strikingly verify and extend biochemical findings in new dimensions. Information thus derived will find application in control of reproduction, growth, differentiation and viral disease in higher organisms.

Publications:

1. Chin, W. and Maizel, J.V.: The Polypeptides of Adenovirus. VII. Further Studies of Early Polypeptides In Vivo and Localization of E2 and E2A to the Cell Plasma Membranes. Virology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00071-04 LMG

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Study of Adenovirus Gene Functions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Westphal	Visiting Scientist	LMG NICHD
OTHER:	H. C. Meissner	Research Associate	LMG NICHD
	P. Neuwald	Staff Fellow	LMG NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study, now in its fourth year, is the analysis of the instruments and controls of Adenovirus gene expression in lytically infected or in transformed cell cultures. Projects under current investigation include (A) the identification of non-structural virus-specific proteins, (B) mapping of Adenovirus messenger RNA by electron microscopy, and (C) the processing of nuclear Adenovirus RNA. Electron microscopy is in part performed in collaboration with the research group headed by Dr. J. V. Maizel, Jr.

PHS-6040
(12-75)

1. Project Description:

A. Identification of non-structural Adenovirus-specific proteins.

This project concerns the structural and functional identity of proteins that mediate Adenovirus gene expression in lytically infected and in transformed animal cells. Established techniques were modified to allow fractionation of cells and viruses under optimally controlled conditions. Protein extracts were analyzed by polyacrylamide gel electrophoresis.

Several virus-specific polypeptides, ranging in size from 11,000 to 72,000 daltons, were detected in extracts of cells harvested at early stages of productive infection with Adenovirus Type 2. A functional analysis of these proteins was initiated by two experiments. First, a time course of appearance of individual polypeptides was established, and related to the time course of viral progeny DNA synthesis. Messenger RNA, coding for these proteins, was translated *in vitro*. Second, the distribution of early virus-specific proteins in the cell's major compartments, nucleoplasm and cytoplasm, was measured. Another protein, likely to be involved in viral DNA synthesis, was found intimately associated with virion DNA. Analysis of the polypeptide chains of the protein in polyacrylamide gels was possible only after exhaustive digestion of the nucleoprotein complex with DNase.

Future efforts will be directed toward the purification of individual non-structural virus proteins. Purified proteins will be examined in suitable cell-free systems and biochemical assays to probe their function. Attempts will be made to raise antibodies against purified proteins. These antibodies would be useful in localizing the corresponding antigen in individual cells and in cell extracts. Moreover, it may be interesting to test the anti-viral effect of such antibodies.

B. Mapping of Adenovirus Messenger RNA by Electron Microscopy. (see also project Number Z01 HD 00070-16 LMG)

The purpose of this study is to determine the location of individual gene functions on the viral DNA. Adenovirus messenger RNA with known informational content was hybridized with viral DNA, and the nucleic acid hybrid structure was visualized by electron microscopy.

Late Adenovirus messenger RNA, containing the genetic information of structural virus proteins, annealed to various regions of the viral DNA. Hybridization occurred most frequently in a region that measured about 10% of the DNA length and was located near the center of the molecule. This is the position of hexon, the most frequent of the components of the virus particle. In contrast, early messenger RNA hybridized predominantly to a DNA region located near the end of the molecule.

The proposed course of this project is to map as many as possible of individual Adenovirus Type 2 messenger RNA's, thereby creating an accurate physical map of the gene functions of this virus.

We further propose to visualize viral DNA sequences integrated in the genome of transformed cells. The DNA of the transformed cells will be fractionated with the help of a restriction enzyme. Fragments containing viral DNA will be hybridized with complementary viral RNA. This procedure should sufficiently alter the physical properties of fragments containing viral DNA to allow their separation from the bulk of cellular DNA. Visualization of these fragments in the electron microscope would thus become possible.

C. Investigation of Adenovirus RNA processing

Adenovirus is an excellent model to study the pathway of controlled RNA degradation from the primary transcript made in the nucleus to the message translated in cytoplasmic polysomes. We have modified existing procedures of RNA extraction from cell nuclei to allow for quantitative preparation of undegraded RNA. The RNA has been characterized by sucrose gradient analysis and by electron microscopy.

We began our analysis with the RNA extracted from nuclei of cells harvested at late stages of lytic infection with Adenovirus Type 2. Samples of RNA that sedimented faster than 45S were examined by electron microscopy. The preparation contains viral RNA that spans almost the entire length (13 μm or 24 x 10^6 daltons) of viral DNA. This RNA is likely to be the precursor of the various species of messenger RNA found in the cytoplasmic polysomes of the same cells.

Forthcoming studies will include: (a) Analysis of the secondary structure of Adenovirus RNA. The nuclear transcripts contain a number of loops that are clearly visible in electron micrographs. We would like to map these in order to use them as markers in the study of RNA processing. (b) Intermediates of RNA processing. Through a combination of biochemical and electron microscopy techniques we hope to detect major pathways of controlled Adenovirus RNA degradation. (c) Control of primary transcription. Nuclear RNA extracted from transformed cells as well as from cells harvested at defined time points after lytic infection will be examined in the electron microscope in an effort to elucidate the regulation of nuclear Adenovirus RNA synthesis.

The described studies of Adenovirus gene functions are in several ways significant for biomedical research and for the program of our Institute. They contribute to our understanding of the molecular events which accompany cell infection by a virus that is known as a human pathogen in childhood diseases, as well as a tumor virus in rodents. At the same time, these studies are likely to offer insights into basic mechanisms of the genetic control and regulation of animal cells. Furthermore, they may help to elucidate some of the principles that govern animal cell transformation.

2. Publications:

Eron, L., Westphal, H. and Khoury, G.: Posttranscriptional Restriction of Human Adenovirus Expression in Monkey Cells. J. Virol. 15: 1256-1261, 1975.

Eron, L. and Westphal, H.: Cell-free Translation Products of Early RNA from Adenovirus Type 2-Infected KB Cells. INSERM 47: 299-304, 1975.

Westphal, H. and Crouch, R.J.: Cleavage of Adenovirus Messenger RNA and of 28S and 18S Ribosomal RNA by RNase III. Proc. Nat. Acad. Sci. USA 72: 3077-3081, 1975.

Westphal, H.: In Vitro Translation of Adenovirus Messenger RNA. Current Topics Immun. Microbiol. 1976, in press.

Westphal, H., Meyer, J.M. and Maizel, J.V., Jr.: Mapping of Adenovirus Messenger RNA by Electron Microscopy. Proc. Nat. Acad. Sci. USA 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00072-09 LMG

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Chick Lens Cell Differentiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joram Piatigorsky	Biologist	LMG NICHD
OTHER:	Peggy Zelenka	Staff Fellow	LMG NICHD (until 9/1/75)
	David C. Beebe	Staff Fellow	LMG NICHD
	Toshimichi Shinohara	Visiting Fellow	LMG NICHD
	Rosalie Reszelbach	Staff Fellow	LMG NICHD

COOPERATING UNITS (if any)

Joseph Horwitz, Ph.D., Jules Stein Eye Institute, U.C.L.A., Los Angeles, California; Robert T. Simpson, National Institute of Arthritis, Metabolism and Digestive Diseases, N.I.H., Behtesda, Md.

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.2	4.2	0

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to describe the molecular events regulating the differentiation of embryonic chick lens cells as a model for understanding the control of embryonic cellular differentiation. Within this past fiscal year, we have 1) quantitated δ-crystallin mRNA during lens cell differentiation, and correlated δ-crystallin gene expression with lens cell elongation; 2) established that the δ-crystallin genes are not amplified during lens cell differentiation; 3) demonstrated that the synthesis of δ-, α-, β- and non-crystallin polypeptides are noncoordinately regulated during lens cell differentiation; 4) made considerable progress on our understanding of the subunit composition and the structure of embryonic δ-crystallin; and 5) initiated experiments directed towards investigations of tubulin synthesis and gene expression during lens cell differentiation.

Project Description:

Objectives: To describe the normal sequence of events at the molecular level during the differentiation of embryonic animal cells. The embryonic chick lens cell has been chosen as a model, since this system displays characteristic changes in cell division, cell shape and specific protein synthesis, three of the basic events regulated during cellular differentiation.

Methods Employed: Conventional techniques for nucleic acid extraction, mRNA purification, mRNA reverse transcription, molecular hybridization, and protein purification and analysis were used. Our approach combines an analysis of embryonic cells at different stages of differentiation in the intact lens with a detailed study of lens fiber cell differentiation in tissue culture. The latter system, largely developed in this laboratory, has been shown to mimic the normal process of lens fiber cell differentiation.

Major Findings: (1) Experiments with δ-crystallin ^{3}H-cDNA showed that δ-crystallin mRNA begins to accumulate in the presumptive lens ectoderm during the initiation of lens placode formation. δ-Crystallin mRNA accumulates at an approximate rate of 1 molecule/lens cell/minute during this inductive phase of lens development. These experiments suggest that the initiation of δ-crystallin synthesis is controlled by the accumulation of δ-crystallin mRNA. (2) δ-Crystallin mRNA was shown to accumulate at an approximate rate of 5-10 molecules/cell/minute during the differentiation of epithelial cells into fiber cells *in vitro*, as well as during the maturation of fiber cells *in vivo* between 6 and 15 days of development. (3) Differential δ-crystallin synthesis and δ-crystallin mRNA were shown to take place during lens fiber cell differentiation in a chemically defined medium containing porcine insulin. (4) Experiments with colchicine showed that the differential synthesis of δ-crystallin and the accumulation of its mRNA can occur in tissue culture in the absence of cell elongation. (5) Annealing experiments with δ-crystallin ^{3}H-cDNA demonstrated that the specialization of δ-crystallin synthesis in lens cells occurs without amplification of the δ-crystallin genes, and that the δ-crystallin genes are situated in the unique fraction of the chick genome. (6) Detailed electrophoretic analyses showed that the differential stimulation of δ-crystallin synthesis during lens fiber cell differentiation *in vitro* is associated with a maintenance in the rate of synthesis of α- and β-crystallins, and a decrease in the rate of synthesis of noncrystallin proteins. (7) Analysis of the methionine-containing tryptic peptides and cyanogen bromide peptides of native δ-crystallin indicated that the 4 subunits composing the protein have very similar, possibly even identical, encoded amino acid sequences. This conclusion was supported by the electrophoretic homogeneity of δ-crystallin mRNA and the hybridization kinetics of δ-crystallin mRNA and its cDNA. (8) Preliminary data suggest that δ-crystallin mRNA translates a precursor polypeptide approximately 10 amino acids longer than the final-sized subunit. (9) Native δ-crystallin was characterized by circular dichroism spectroscopy, and shown to contain a great deal of α-helix secondary structure. This is unique to δ-crystallin, since all other lens crystallins have a β-pleated sheet structure. (10) The subunits of δ-crystallin were partially dissociated in urea and in guanidine hydrochloride and, for the first time, renatured. Circular dichroism and ultracentrifugation tests showed that the subunits reconstituted a tetramer with its characteristic tertiary structure. (11) A comparison of chick and bovine α-crystallin revealed that the subunits from the chick protein differ from those of the bovine protein. This represents the first unambiguous species difference between the subunits of α-crystallin. (12) Finally, tubulin mRNA has been partially purified from embryonic chick brain and translated in vitro.

Significance to Biomedical Research and the Program of the Institute: These studies contribute to our knowledge of the mechanisms of cellular differentiation and, especially, to specific protein synthesis during development. In addition, the detailed analysis of the lens crystallins relate to an understanding of the function of the eye lens and its role in refracting light and thus, ultimately, add to our knowledge of cellular transparency and the cause of the loss of such transparency, as occurs in lens cataracts.

Proposed Course of Project: The differentiation of embryonic chick lens cells will be studied further. Emphasis will be placed on the metabolism of δ-crystallin mRNA during lens cell differentiation, the synthesis and composition of δ-crystallin, the relationship between δ-crystallin synthesis and lens cell elongation, and the acquisition of a tubulin cDNA probe.

Publications:

1. Piatigorsky, J.: Post-translational modification of δ-crystallin in the embryonic chick lens. Exp. Eye Res. 21: 245-250, 1975.

Piatigorsky, J.: Lens cell elongation and microtubules. Ann. N.Y. Acad. Sci. [illegible]: [illegible]-347, 1975.

Piatigorsky, J.: Gametogenesis. In Czihak, G. (Ed.): The Sea Urchin Embryo. Berlin, Springer-Verlag, 1975, pp 42-98.

Milstone, L.M., Zelenka, P. and Piatigorsky, J.: δ-Crystallin mRNA in chick lens cells: mRNA accumulates during differential stimulation of δ-crystallin synthesis in cultured cells. Develop. Biol. 48: 197-204, 1976.

5. Zelenka, P. and Piatigorsky, J.: Molecular weight and sequence complexity of δ-crystallin mRNA. Exp. Eye Res. 22: 115-124, 1976.

6. Beebe, D.C. and Piatigorsky, J.: Differential synthesis of crystallin and noncrystallin polypeptides during lens fiber cell differentiation in vitro. Exp. Eye Res. (in press).

7. Beebe, D.C. and Piatigorsky, J.: Electrophoretic differences between calf and embryonic chick lens α-crystallin polypeptides. Exp. Eye Res. (in press).

8. Piatigorsky, J. Beebe, D.C., Zelenka, P., Milstone, L.M. and Shinohara, T.: Regulation of δ-crystallin gene expression during development of the embryonic chick lens. Colloques et Seminaires de L'I.N.S.E.R.M. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00073-05 LMG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Antibody Response at the Cellular Level

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edgar E. Hanna	Microbiologist	LMG NICHD
OTHERS:	Martha L. Hale	Staff Fellow	LMG NICHD

COOPERATING UNITS (if any)

C.T. Hansen, Geneticist, VR DRS, NIH

LAB/BRANCH
Laboratory of Molecular Genetics

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	2.0	0.25

SUMMARY OF WORK (200 words or less - underline keywords)

Studies are directed toward understanding the process by which regulation of the immune system is controlled at the cellular level. The co-operative roles of thymus derived (T) cells, both helper T-cells and suppressor T-cells are being delineated. We are seeking an understanding of the mechanism of interaction of these cells. We have shown that a streptococcal exotoxin (SPE) inhibits preferentially an activity which is presumed to be controlled by suppressor cells. SPE might therefore be a useful probe for the study of suppressor cells. Other standard reagents such as bacterial endotoxins, and various plant lectins such as phytohemagglutinins (PHA) are used to identify precursors of antibody secreting cells (B-cells) and helper T-cells respectively. Further cell separations are made by selective cytolysis with specific antisera and Guinea-pig-complement (C') and by various adherence techniques. Immunocytes from immunocompetent (+/nu) donor mice selected in this way are tested in reconstruction experiments for their capacity to complement the immune responses of T-cell deficient Nude (nu/nu) mice or their spleen immunocytes in vitro. This study involving the use of SPE may also derive relevant information for a solution of the sequelae of Streptococcosis.

Objectives:

To understand the process by which regulation of the immune system is controlled at the cellular level. Specificially, we seek to understand the mechanism by which macrophages and thymus-derived cells (T-cells) regulate antibody formation and secretion by interaction with precursors of antibody forming cells (B-cells). We wish to understand the relationship between helper T-cells which augment B-cell responses, and suppressor T-cells which curtail B-cell responses. It remains unclear whether these are different T-cells or only apparently different because of the expression of variable activities in response to different stimuli. We seek to understand the physical mechanism of the interactions between T-cells and B-cells. This interaction, as well as that with macrophages may involve products of the histocompatibility (H2) locus.

Methods Employed:

These were detailed in previous reports. Major advantage is taken of our ability to do reconstruction experiments in vitro. These involve the use of nude mouse B-cells and macrophages to which we can add variously tested donor T-cells, then ask whether complete antibody responsiveness is conferred upon B-cells from nude mice. Similarly, macrophages may be separated from nude cell populations, treated variously, withheld or added back to ask similar questions about the macrophage as asked for T-cells.

Major Findings:

Relative to distinguishing between helper cells and suppressor cells, we observe that the anti-suppressor activity of SPE is obtained without effect on helper cell activity. Similarly, the synergistic activity of macrophages has been distinguished as a separate activity. The actual localization of these activities to a cellular site and delineation of target of SPE awaits the results of the physicial binding studies outlined above.

In the course of our studies we discovered that nude mice borne by homozygous nude mothers are even more T-cell deficient than the heretofore available nude mice borne and raised by heterozygous, T-cell sufficient mothers. We demonstrated that these nude mice may receive residual T-cell helper activity from heterozygous mothers. Whether such helper activity is conferred by passive transfer of T-cells, or soluble T-cell factors, or factors which stimulate the maturation of T-cell precursors remains to be distinguished. T-cell precursors have been demonstrated in other nude mice by other investigators. Such precursors have not been searched for in our nude mice.

Significance to Biomedical Research and the Program of the Institute:

Understanding the natural mechanism of the regulated expressions of the immune system may reveal pathways by which defective immune systems may be corrected. The potential ability to deregulate and to re-organize individual immune systems is highly relevant to cures for certain diseases. SPE is known to be a metabolite of only the pathogenic Group A streptococci. Our demonstration that SPE can deregulate immune systems may be relevant to an understanding of the mechanism of certain autoimmune sequelae of acute streptococcal infections. Furthermore results of our study should complement scientific thought on cellular control mechanisms in general.

Proposed Course of Project:

We will test for selective binding of SPE by certain immunocytes with anticipation of identifying suppressor cells by immunofluorescence. Anti-SPE-specific antiserum is being prepared for this purpose. Antisera to other important lectins, which show preferential binding to other cell types of interest have been prepared or have been obtained otherwise other reagents are available commercially.

We will carry out experiments to distinguish between three possible paragenetic modes by which mutant T-cell deficient nude mice receive T-cell activity maternally. This was discussed above under major findings.

The most productive means for conducting these studies is immunofluorescence, using an incident light fluorescence microscope system. This system includes an electronic photographic component which optimizes conditions to visualize discrete structures of interest within and on the surface of cells. We have requested the purchase of the best available equipment for this purpose. It is obvious that reagents such as antisera must be well characterized and highly specific. We will devote some effort toward this requirement as the experiments proceed.

Publications:

. Hanna, E.E. and Hale, M.L.: Deregulation of Mouse Antibody-Forming Cells by a Streptococcal Exotoxin - A Model Study of the Regulation of Antibody Formation by Spleen Cells. In Battisto, J.R. and Streilein, J.W. (Eds): *Immuno-Aspects of the Spleen*. Amsterdam, Elsevier/North-Holland Biomedical Press B.V., 1976, In press.

2. Hale, M.L., Hanna, E.E. and Hansen, C.T.: Nude Mice from Homozygous Nude Parents Show Smaller PFC Responses to Sheep Erythrocytes than Nude Mice from Heterozygous Mothers. *Nature*, 260: 44-45, 1976.

3. Lambert, W.C. and Hanna, E.E.: Origin of Lymphoid Cell Independent Plaques (LCIP) in Rabbit PFC Cultures. *Proc*. *Soc*. *Exp*. *Biol*. *Med*. 150: 636-640, 1975.

4. Lambert, W.C. and Hanna, E.E.: Characteristics of the Artibody Response of Rabbit Spleen Cells In Vitro. *Cell*. *Immunol*., In press, 1976.

Research Reports at Special conferences, Annual Scientific Meetings and as Guest Lecturer.

1. Hanna, E.E. and Hale, M.: Deregulation of Mouse Antibody-Forming Cells by a Streptococcal Exotoxin - A Model Study of the Regulation of Antibody Formation by Spleen Cells. First Conference on Immuno-Dynamics; Immuno-Aspects of the Spleen held at the Dept. of Immunology, Research Division, Cleveland Clinic Foundation and Cleveland Clinic Educational Foundation May 19-20, 1976.

2. Hale, M.L. and Hanna, E.E.: Deregulation of T-cell Complemented Nude Mouse PFC Responses by SPE. F.A.S.E.B. Annual Meeting, Anaheim, CA. April 11-16, 1976.

3. Hanna, E.E.: Deregulation of Antibody-Forming Spleen Cells of the Mouse by a Streptococcal Exotoxin - A Potential Probe for Regulatory T-Cells. Invited Speaker Research Division, Dept. of Pediatrics, University of Alabama in Birmingham, Dec. 13, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00074-05 LMG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Regulated Gene Expression During Cell Growth and Differentiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Leder	Chief	LMG NICHD
OTHERS:	T. Honjo	Visiting Fellow	LMG NICHD
	S. Orkin	Research Associate	LMG NICHD
	D. Swan	Visiting Associate	LMG NICHD
	D. Tiemeier	Staff Fellow	LMG NICHD

COOPERATING UNITS (if any)
Department of Biological Chemistry, Hebrew University, Jerusalem, Israel

LAB/BRANCH
Laboratory of Molecular Genetics

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.75	1.75	5.5

SUMMARY OF WORK (200 words or less - underline keywords)

Our objective is to define in genetic and molecular terms the mechanisms which regulate the expression of genetic information during cell growth and development. For this purpose, we have investigated two model systems which serve as analogs for erythrodifferentiation and immunodifferentiation. This work has led to development of techniques for gene isolation, has defined the sequence of the expression of the α and β globin genes, has strengthened the somatic mutation hypothesis as a mechanism for generating immunoglobulin diversity and has led to the construction of safer vectors for use in the cloning of recombinant DNA molecules.

PHS-6040
(12-75)

Project Description:

Objectives: Our objective is to understand, in molecular terms, the mechanisms which regulate the flow and exchange of genetic information during cell growth and development. We use two model systems in these investigations, one involving the process of erythrodifferentiation, the other immunodifferentiation. Specifically, we ask how the genetic elements corresponding to globin and immunoglobulin are organized in the genome, how they arose during evolution, what elements in the genome and what gene products regulate their expression and how this regulation and organization is relevant to other elements of the genome of higher organisms.

Methods Employed: These investigations have, naturally, involved an interdisciplinary approach. Each model system involves either the growth in culture or animal hosts of tumor cells, the isolation and purification of specific mRNAs, their reverse transcription using avian myeloblastosis virus RNA-dependent DNA polymerase, the application of hybridization kinetic analyses, techniques for the specific characterization of tryptic peptides, electron microscopic analysis and the development and application of special techniques for the containment of potentially biologically hazardous materials. Among the techniques which have been specifically developed in this laboratory are affinity chromatographic techniques for the isolation for specific mRNAs as well as for the isolation of specific sequences from genomic DNA.

Major Findings:

1. Experiments early in the year led to the completely unexpected discovery that butyric acid, a reagent commonly used to derivatize cyclic AMP, would induce the process of erythrodifferentiation in cultured erythroleukemic cells. The discovery of the biologic effectiveness of this reagent, while significant for research involving dibutyrl cyclic AMP, presented an additional advantage in that it afforded a relatively nontoxic reagent which would induce erythrodifferentiation in cultured cells.

2. Using butyric acid we were able to conclusively demonstrate that DNA replication was not required for the process of erythrodifferentiation, thus enormously simplifying the models required to explain the process of globin gene regulation.

3. Studies involving the origin of immunoglobulin diversity were extended and the genetic representation of the variable region gene sequence was assessed quantitatively. The variable sequence of the λ gene appears relatively unique by hybridization kinetic analysis, thus strengthening the notion that immunoglobulin diversity arises through a process of somatic mutation.

4. Inasmuch as the technology that has arisen through the cloning of recombinant DNA molecules is potentially of great use in these studies we also participated in the development of a λ phage cloning vector which has properties making it quite amenable to biological containment.

Significance to Biomedical Research and the Program of the Institute: These studies are directed towards understanding fundamental processes involved in the expression of genetic information. They are, thus, obviously relevant to achieving an understanding of a variety of inherited disorders that afflict man. By inherited disorders we refer not only to clearly identifiable conditions which involve mutations at a single genetic site, but also polygenic disorders such as diabetes, hypertension and susceptibility to oncogenic disease. For example, our studies in the past have led to techniques which have permitted a far more detailed understanding of the inherited anemia, thalassemia. Current studies involving the construction of safe vectors for gene cloning are obviously relevant to the possible production of human polypeptide hormones.

Proposed Course of Research: Efforts over the next year will focus on understanding the organization of globin and immunoglobulin genes. Assuming the approval of safe biologic containment facilities we intend to clone this genetic sequence in appropriate vectors, to determine their structural and regulatory sequences, to test cardinal models of genetic evolution and to begin chromosome reconstruction studies.

Publications:

1. Leder, P., Honjo, T., Swan, D., Packman, S., Nau, M. and Norman, B.: The Organization and Diversity of Immunoglobulin κ and λ Genes. In Smith, E.E. and Ribbons, D.W. (Eds.): Molecular Approaches to Immunology. Academic Press, Inc., New York, 1975, 9: 173-188.

2. Leder, A. and Leder, P.: Butyric Acid, A Potent Inducer of Erythroid Differentiation in Cultured Erythroleukemic Cells. Cell 5: 319-322, 1975.

3. Leder, A., Orkin, S. and Leder, P.: Differentiation of Erythroleukemic Cells in the Presence of Inhibitors of DNA Synthesis. Science 190: 893-894, 1975.

4. Orkin, S., Swan, D. and Leder, P.: Differential Expression of Alpha and Beta Globin Genes During Differentiation of Cultured Erythroleukemic Cells. J. Biol. Chem. 250: 8753-8760, 1975.

NICHD Annual Report
July 1, 1975 through June 30, 1976
Neonatal and Pediatric Medicine Branch

The Neonatal and Pediatric Medicine Branch conducts basic and clinical research on lung and liver development; nutrition in infants, especially those of low birth weight and with disorders of somatic growth; calcium metabolism and transport in the newborn; endocrine function in infants and children, amino acid transport; and metabolic pathways in various inborn errors of metabolism.

In the Section on Developmental Biology and Clinical Nutrition, interests center on the regulatory mechanisms involved in promoting maturation of fetal and neonatal organs, with emphasis on biochemical development of lung in relation to synthesis of surface active choline phosphoglycerides (surfactants). Various hormones such as cortisol and thyroxin are studied with respect to their effect on lung differentiation. Other areas of major interest include metabolic and nutritional aspects of growth and development in infants, states of malnutrition associated with prematurity and intestinal malabsorption syndromes, mineral metabolism and hypocalcemia in the newborn, and endocrine function occurring between conception and the atainment of adulthood.

It has become increasingly clear in recent years that a decrease in the content of lung surfactant (composed primarily of highly saturated phosphatidylcholine) is the underlying mechanism in the disorder of newborns termed respiratory distress syndrome. Although the precise etiology of this disorder is not completely understood, metabolic abnormalities affecting phospholipid biosynthesis are highly suspect. The principal mechanism for lung phosphatidylcholine (PC) synthesis has been identified as the choline incorporation pathway rather than the other de novo route, phosphatidylethanolamine methylation. Conversion of choline to the phosphoglyceride involves three enzymic reactions: (1) phosphorylation of choline, (2) activation of phosphorylcholine to the cytidine diphosphocholine intermediate, and (3) incorporation of phosphorylcholine into 1, 2-diacylglycerol. New, rapid and accurate methods have been developed for evaluation of the enzymes in this pathway. An especially useful technique has been devised for measuring choline kinase, the first enzyme of the pathway and a possible regulator of PC synthesis. Pathways and enzymes for production of phosphatidylcholine have been identified in tissue cultured lung cells which morphologically resemble the type II pneumonocytes of the alveolar epithelium, thought to be the sources of lung surfactant. These cells are highly enriched in choline pathway enzymes and show greater PC synthetic activity than any source identified to date.

The role of cortisol and thyroxin in controlling biochemical development of the lung has been assessed under a variety of conditions. Lung PC concentration and its rate of synthesis have been shown to increase during the latter 10-20% of gestation in many species. Increased choline pathway enzyme activities have been demonstrated after injection of the animals with either cortisol or thyroxin. A multidisciplinary approach involving techniques of biochemistry, physiology, and histology has been directed toward correlating biochemical changes in lung tissue with functional and anatomic differentiation. Pressure-volume hysteresis and the content of surface active material have been measured in lung samples in developing monkey fetuses and found to agree with biochemical evidence of maturation. It has also been found that development of the fetal primate lung proceeds in an asymmetric fashion with the upper lobes attaining maturity first. Studies with amniotic fluid phospholipids have shown that increased levels in late gestation correlate well with the physiologic biochemical state of lung maturity in the Rhesus monkey fetus. Thus, measurement of amniotic fluid phospholipids provides a specific and direct index of the state of fetal lung maturity and permits prenatal identification of infants at risk for respiratory distress syndrome.

In addition to promoting lung differentiation, administration of corticosteroids to pregnant monkeys has been found to accelerate the development of other organs including liver and kidney. Marked increases in hepatic glycogen levels were demonstrated within 48 hours after maternal administration of betamethasone. The effect of antenatal corticosteroid administration on fetal liver glycogen stores has been extended to an animal model of intrauterine growth retardation. Studies with fetuses of pregnant rats subjected to surgically-induced placental insufficiency have confirmed previous observations of reduced liver glycogen. This work has further revealed a high incidence of hypoglycemia in the growth retarded offspring when they are deprived of postnatal nutrition for two to six hours. Antenatal treatment with dexamethasone was found to increase liver glycogen levels in the growth retarded neonatal rats and prevent prolonged hypoglycemia.

Endocrine studies have shown for the first time that a monthly gonadotropin pattern exists prior to menarche in young women and at least in some girls prior to any signs of puberty. Investigation of selected aspects of neonatal hypocalcemia indicates that calcitonin may be active in the fetus and newborn accentuating any tendencies to low blood calcium and seizures in the newborn. Measurement of ionized calcium levels in a limited number of cerebrospinal fluid samples suggests that seizure activity associated with hypocalcemia may occur only after Ca^{2+} levels fall in cerebrospinal fluid.

With respect to the regulation of fetal lung surfactant synthesis, further attempts will be made to purify choline kinase and study its physical-chemical properties. The kinetic characteristics of this catalyst will then be examined and promising leads suggesting that the purified enzyme can be activated *in vitro* by effector molecules will be pursued. Using lung cells in tissue culture, factors influencing the activities of choline pathway enzymes and phosphatidylcholine biosynthesis will be investigated. The effects of cortisol and thyroxin on phospholipid metabolism will be evaluated with these cells. Basic studies will be also carried out to define the metabolic characteristics of such cells under various culture conditions. Studies with the rat model of intrauterine growth retardation will be pursued with particular attention directed toward assessment of plasma amino acid levels and gluconeogenesis. It is planned to determine the hepatic activities of phosphoenolpyruvate carboxykinase and tyrosine aminotransferase in growth retarded rats, as well as in cortisol treated animals.

Work will continue on characterizing the nutritional status of infants and children with disorders compromising the assimilation or utilization of key dietary elements. Neonatal calcium metabolism will be studied using animals to establish the role of cerebrospinal fluid ionized calcium in hypocalcemic seizures. Determination of calcium kinetics in infants using stable, non-radioactive tracers will be actively pursued and should produce data permitting development of a rational approach to diagnosis and treatment of neonatal disorders involving calcium. Studies of trace minerals in newborns, older children, and adults receiving total parenteral nutrition will be pursued. Gonadotropin patterns and the dynamics of pubescence will be further studied in prepubertal and pubertal females.

The Section on Human Biochemical and Developmental Genetics investigates human inborn metabolic disorders, with special emphasis on cystinosis and other defects in sulphur amino acid metabolism. Exploration of the molecular basis of these diseases and development of new therapeutic modalities are major research goals. Other areas of interest include: cellular amino acid transport, lysosomal structure and function, role of amino acids in fetal and neonatal nutrition, *in vitro* fertilization of mammalian oocytes, the biochemistry of early embryonic development, and the study of endocrine and biochemical factors which regulate the metabolism of ovarian cells *in vitro*.

Two patients have been discovered with inborn errors of metabolism characterized by an inability to break down glutathione, i.e., gamma-glutamyl transpeptidase deficiency. Using cells from these patients, it has been possible to test the hypothesis advanced by others that this enzyme is involved in mammalian amino acid transport. Results indicate that in the absence of such enzyme activity, intracellular amino acid transport kinetics and pool sizes remain normal. Studies of numerous human cultured fibroblast lines and organs from developing rats have demonstrated the importance of gamma glutamyl transpeptidase in regulating intracellular glutathione content. It has been demonstrated that children with oxyprolinuria, manifested by acidosis and a marked reduction in glutathione synthase activity in red cells and cultured fibroblasts, have genetic alterations in the synthase enzyme. In contrast, patients with adult form of glutathione synthase deficiency without acidosis have an unstable synthase resulting in selective expression of their metabolic defect in red cells. Work on glutathione synthase in normal and mutant tissues and various cells has indicated that different forms of the enzyme exist.

It has been shown that cysteamine may be enclosed in liposomes. Exposure of cystinotic cells to this enclosed sulfhydryl agent leads to additional therapeutic effects, as compared to treatment with "free" cysteamine. Furthermore, radioactive cysteamine enclosed in liposomes distributes more favorably in the rat model than does "free" cysteamine suggesting therapeutic advantages of liposome-enclosed agents in human cystinosis. A study of ascorbic acid treatment in patients with cystinosis is continuing and now encompasses approximately 50 patients. Studies on platelet survival and platelet ultrastructure in homocystinuria have indicated that previous reports of abnormalities are erroneous and thus do not explain the frequent development of thromboembolism in this disease.

Granulosa cells from the estrogen-primed, hypophysectomized rat have been cultured successfully and are being studied in terms of growth rate and production of steroid hormones. The response of plasma amino acid patterns in men and in the newborn monkey during intravenous hyperalimentation has been studied and alterations in plasma amino acids were revealed.

Studies will continue on inborn errors of metabolism. In particular, glutathione and sulphur metabolism in cystinosis will be of major concern. Work will also continue on the metabolic effects of liposome-enclosed sulfhydryl agents and their possible therapeutic role in cystinosis. Other new or planned programs include: (1) an attempt to develop an *in vivo* radiochemical assay for liver phenylalanine hydroxylase activity in patients with phenylketonuria and (2) expansion of interest in patients with inborn errors of metabolism, particularly those involving amino acids.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00126-02 NPMB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Developmental Biology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Farrell	Chief, DBCN Section	NPMB, NICHD
OTHER:	R.E. Ulane	Sr. Staff Fellow	NPMB, NICHD
	R.L. Manniello	Clinical Associate	NPMB, NICHD
	A.J. Adams	Biological Lab. Tech.	NPMB, NICHD
	L.L. Stephenson	Biological Lab. Tech.	NPMB, NICHD
	M.S. Millen	Student Scientist	NPMB, NICHD

COOPERATING UNITS (if any)

R.A. Chez, Pregnancy Research Branch, NICHD
R.K. Kotas, Warren Research Center, Tulsa, Oklahoma
W.H. Douglas, Alton Jones Cell Science Center, Lake Placid, New York
I.S. Wertheimer, Children's Hospital, N.M.C., Washington, D.C.

LAB/BRANCH

Neonatal and Pediatric Medicine Branch

SECTION

Developmental Biology and Clinical Nutrition

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	3.0	3.0

SUMMARY OF WORK (200 words or less - underline keywords)

Lung surfactant production is being studied by assessment of its principal phospholipid component, phosphatidylcholine (PC). The primary mechanism for lung PC synthesis in the fetus has been identified as the choline pathway. Tissue cultured lung cells have been studied and found to show marked enrichment in choline pathway enzymes and greater rates of phospholipid synthesis than any source studied to date. Lung PC concentration has been shown to increase during the latter 10-20% of gestation in many fetal species. Increased choline pathway enzyme activities accompany this change and may be induced by injection of animals with either cortisol or thyroxin. Pressure-volume hysteresis, deflation stability, and the content of surface active material have been evaluated in lungs from developing monkey fetuses and found to change in accordance with the biochemical events of maturation. In addition to promoting lung differentiation, administration of corticosteroids to pregnant monkeys has been found to accelerate the development of other organs including liver and kidney. Marked increases in hepatic glycogen levels were demonstrated within 48 hours after maternal administration of a cortisol analog. Thus, corticosteroids may provide the stimulus for maturation of many fetal organs. Promising data from clinical trials elsewhere suggest that these agents may be useful in the prevention of the respiratory distress syndrome of human neonates.

Project Description:

Objectives: Studies on organ development have focused on fetal and neonatal lung as related to the respiratory distress syndrome (RDS) of human neonates. This disorder occurs in low birth-weight infants, is characterized by progressive pulmonary atelectasis, and accounts for greater mortality and morbidity than any other neonatal disorder or pediatric pulmonary disease. It has become clear in the past several years that a decrease in the concentration of lung surfactant (composed primarily of highly saturated phosphatidylcholine) is of importance in the development of RDS. By inference, a decreased rate of pulmonary surfactant production is the likely pathogenetic factor. In order for the neonate to avoid respiratory insufficiency, it appears essential that the fetal lung in late gestation increase its capability for phosphatidylcholine (PC) synthesis and elevate the concentration of stored surfactant in type II alveolar pneumonocytes. Thus, pulmonary phospholipid metabolism in the fetus must be carefully controlled such that acceleration is initiated at the proper time in gestation. Accordingly, our studies have been directed toward elucidating: (1) the relative contributions of the two pathways for de novo PC synthesis; (2) the gestational timing of increased pulmonary surfactant; and (3) biochemical mechanisms by which PC production is regulated. Investigations are being carried out with various lung preparations including the intact organ, tissue slices, homogenates, and cells in tissue culture. The key question of what factors regulate phospholipid metabolism is being approached by studies on the enzymes and substrates of the relevant pathways. The role of hormones such as cortisol and thyroxin in controlling biochemical development of the lung is being assessed in various models.

Methods Employed: PC synthesis is measured using isotopic techniques in conjunction with thin layer chromatography. Activities of the enzymes of the choline pathway are measured by newly developed techniques using labelled substrates and chromatographic methods of product isolation. Animals have been injected with cortisol analogs or thyroxin and evaluated after various periods of time with respect to PC synthesis and enzyme activities. Differentiated lung cells showing characteristic osmiophilic lamellar bodies have been cultured and studied biochemically.

Major Findings: (1) The principal mechanism for lung phosphatidylcholine synthesis has been identified as the choline pathway rather than the other de novo route, phosphatidylethanolamine methylation. New, rapid and accurate methods have been developed for evaluating enzymes of the choline pathway, particularly the first catalyst, choline kinase. Pathways and enzymes for PC synthesis have been identified in tissue cultured lung cells. These cells are highly enriched in choline pathway enzymes and show greater PC synthetic activity than any source studied to date. Mechanisms for restructuring newly synthesized phosphatidylcholine, which involve phospholipase A and lysolecithin acyltransferase, have been identified and partially characterized in primate lung. Lung PC concentration and its rate of synthesis have been shown to increase during the latter 10-20% of gestation in many species. Increased choline pathway enzyme activities have been demonstrated after injection of animals with either cortisol or thyroxin. A multidisciplinary approach has been directed toward correlating biochemical changes in lung tissue with functional and anatomic differentiation. Pressure-volume hysteresis, deflation stability, and the content of surface active material have been evaluated in lungs from developing monkey fetuses and found to change in accordance with the biochemical events of maturation. It has also been found that development of the fetal primate lung proceeds in an asymetric fashion with the upper lobes attaining maturity first. Studies with amniotic fluid phospholipids have shown that increased levels in late gestation correlate well with physiologic and biochemical indices of lung maturation in the Rhesus monkey fetus. Thus, methods presently in use for the clinical detection of fetal lung maturity by measurement of amniotic fluid phospholipids seem to provide a direct index of the state of the pulmonary surfactant system. (2) In addition to promoting lung differentiation, administration of corticosteroids to pregnant monkeys has been found to accelerate the development of other organs including liver and kidney. Marked increases in hepatic glycogen levels were demonstrated within 48 hours after maternal administration of the potent cortisol analog, betamethasone. Studies on the effect of antenatal corticosteroid administration relative to fetal liver glycogen stores have been extended to an animal model of intrauterine growth retardation in which the offspring are prone to development of hypoglycemia. Evaluation of fetuses delivered from pregnant rats subjected to surgically-induced placental insufficiency have confirmed previous observations of reduced liver glycogen. Antenatal treatment with dexamethasone was found to increase liver glycogen levels in the growth retarded neonatal rats and prevent prolonged hypoglycemia.

Significance to Biomedical Research and the Program of the Institute. Prior to 1971, despite the overwhelming importance of the respiratory distress syndrome as a public health problem, very little was known in general about lung biochemistry and in particular about the synthesis of surface active phosphatidylcholine by fetal and newborn lung tissue. In order to study the metabolic disturbances leading to this disease, basic features of phospholipid metabolism in lung must be identified, particularly those factors which play a regulatory role. Our studies thus far have indicated that the choline pathway is principally responsible for the synthesis of surface active phosphatidylcholine. Increases in the rate of this pathway in late gestation correlate with development of pulmonary functional maturity. Corticosteroids and thyroxin have been identified as agents capable of promoting lung PC synthesis, an action seemingly mediated by enzyme induction. Promising data from clinical trials elsewhere suggest that these pharmacologic agents may be useful in prevention of neonatal respiratory distress syndrome.

Proposed Course of Project: Attempts will be made to purify choline kinase and study its physical-chemical properties. The kinetic characteristics of this catalyst will then be examined and promising leads suggesting that the purified enzyme can be activated *in vitro* by effector molecules will be pursued. Using lung cells in tissue culture, factors influencing the activities of choline pathway enzymes will be investigated.

Basic studies will also be carried out to define the metabolic characteristics of such cells under various conditions of culture. Regarding the rat model of intrauterine growth retardation, plasma amino acid levels will be determined and gluconeogenesis evaluated before and after antenatal corticosteroid treatment.

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2. Epstein, M.F., Farrell, P.M., and Chez, R.A.: Lung lecithin synthesis in primates as related to respiratory distress syndrome. In Goldsmith, E.I., and Moor-Jankowski, J. (Eds.): Advances in Medical Primatology, Basek, Karger: New York in press.
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5. Epstein, M.F., and Farrell, P.M.: The choline incorporation pathway: primary mechanism for de novo lecithin synthesis in fetal primate lung. Pediatr. Res., 9: 658, 1975.
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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00127-02 NPM B

PERIOD COVERED

July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Developmental Endocrinology and Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator:

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H. J. Hoffman, Biometry Branch, NICHD
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LAB/BRANCH

Neonatal and Pediatric Medicine Branch

SECTION

Section on Developmental Biology and Clinical Nutrition

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Md., 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
10/12	9/12	1/12

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying endocrine and metabolic changes which occur as an individual develops from a fetus to a mature adult. We have currently focused on two areas: 1) monthly gonadotropin cycles in premenarchial girls and pubescent boys utilizing radioimmunoassay methods for measuring urinary luteinizing hormone and urinary follicle stimulating hormone and analyzing the data with autoregressive computer techniques, and 2) mineral metabolism in the monkey fetus and human newborn. Calcium kinetics are studied using stable calcium isotopes in the patient analyzed in the laboratory by mass spectrometry. Physiological effects of calcium are studied by measurements of ionized calcium in the blood and in cerebrospinal fluid. Trace element (zinc and copper) analyses are performed by atomic absorption spectrophotometry as part of our studies of nutritional aspects of mineral metabolism in the newborn.

Project Description:

Objectives: We are studying the dynamics of pubescence from several aspects. Urinary hormone (gonadotropin) excretion patterns around the time of puberty are being determined to understand better the process of pubescence and reproductive cycles in man. Clinical parameters of skeletal metabolism are also being studied in the same subjects to correlate these with the process of pubescence during which ultimate height is established. Similar studies of hormonal function and calcium metabolism and kinetics along with studies of trace element requirements are also being undertaken in the period around birth to understand better and to aid the newborn infant in its adjustment to extrauterine life. A clinical unit, the special care nursery, is being supported to enable these and other clinical projects. The role of ionized calcium in cerebrospinal fluid, especially as it relates to hypocalcemic seizures, is also being investigated.

Methods Employed: Radioimmunoassay of gonadotropins; computer analysis of the data using autoregressive filtering techniques; urinary hydroxyproline and serum alkaline phosphatase, calcium, phosphorus, magnesium, zinc, copper, total protein, quantitative albumin, pH, and ionized calcium measurements; calcium kinetic studies using stable isotopes and noninvasive urinary collection techniques for sample acquisition in newborn infants; mass spectroscopy of calcium isotopes; prepartum studies in nonhuman primates by cannulation of the interplacental vessel.

Major Findings: To date, 43 persons have participated in the study of pubescence and specimens have been completely analyzed from 14 of the girls. These data have disclosed for the first time monthly hormonal (gonadotropin) cycles before the onset of menstrual periods. Preliminary results from the skeletal metabolism measurements reveal a marked decline in hydroxproline and alkaline phosphatase values during pubertal stage IV, just after menarche. These observations are consistent with the decline in skeletal growth after puberty. Preliminary data also suggest that calcitonin is an active hormone in the fetus, perhaps enhancing skeletal accumulation of calcium in the last trimester of pregnancy. We have also observed in the rat that lowering serum calcium does induce seizures, but not until hypocalcemia has been present for a considerable period, suggesting that a lowering of brain or cerebrospinal fluid calcium may be necessary before hypocalcemia results in convulsions.

Significance to Biomedical Research and the Program of the Institute: The finding of monthly hormonal cycles in girls before puberty suggests that there may be an important cyclic feedback mechanism operative in girls even before menstrual periods begin. These studies of the development, onset, and mechanism of reproductive function will help us understand and treat disorders of pubescence and perhaps enable better control of fertility and sterility according to the needs of the patient. Understanding the relationship of skeletal metabolism to the process of pubescence and growth will provide useful clinical parameters to monitor the growth process and potential during the critical period of puberty when final height is established. During the period around birth, calcium is accumulated in the rapidly growing skeleton of the fetus. With delivery, the newborn is cut off from his rich supply of calcium via the placenta and is subject to hypocalcemia and seizures. This is particularly true of the premature infant and the results of our studies will finally provide a rational basis for diagnosis and treatment of these infants with nutritional and metabolic problems relating to calcium. These studies of the dynamics of pubescence and of developmental endocrinology and metabolism in the period around birth have a direct bearing on the mission of the Neonatal and Pediatric Medicine Branch and of NICHD to understand better the process of growth and development and the disorders which arise in the developing person.

Proposed Course of Project: The balance of the specimens which have been collected from the subjects around the time of puberty will be analyzed to establish further the observations in girls and to investigate the possibility that male patterns also exist. Steroid measurements will also be made on the specimens to study possible relationships to the gonadotropin values as our initial attempts to investigate these cyclic phenomena. Our research is progressively placing more emphasis on endocrinology and metabolism around the time of birth with initial studies being directed toward calcium control and metabolism in the fetus and newborn. We are also preparing to investigate trace element metabolism in the newborn as another aspect of clinical nutrition and developmental metabolism which requires elucidation to improve the quality of life to be experienced by premature or malnourished infants.

Publications;

1. Hansen, J.W., and Ross, G.T.: A new method simplifying collection of serial specimens for gonadotropin determinations, J. Clin. Endocr. Metab. 41:241-244, 1975.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00131-02 NPMB

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Human Biochemical Genetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

Neonatal and Pediatric Medicine Branch

SECTION

Human Biochemical and Developmental Genetics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
54/12	42/12	12/12

SUMMARY OF WORK (200 words or less - underline keywords)

Our studies during this year have concerned the metabolic errors of glutathione synthesis and degradation (glutathionuria, glutathione synthase deficiency and gamma glutamyl-cysteine synthase deficiency), cystinosis, homocystinuria, and hyperglycinemia. Patients studied with the abnormalities of glutathione synthase include patients with severe acidosis (oxoprolinuria) and the adult, non-acidotic form of this disorder. We have demonstrated that glutathionuric patients have a deficiency of the enzyme gamma glutamyl transpeptidase but appear to have normal amino acid transport in fibroblasts and kidney despite this enzyme deficiency. The molecular lesions in the acidotic and non-acidotic forms of glutathione synthase deficiency has been distinguished and partially characterized. Our studies of cystinosis have revealed a consistent defect in thyroid function and a controlled clinical trial of high dose vitamin C in this lethal disorder is continuing. Cystinotic fibroblasts have been also treated with liposome enclosed agents as a model for improving drug treatment of cystinosis with promising results. We have studied the kinetics of platelet metabolism and platelet ultrastructure in homocystinuria and have documented normal parameters in six patients despite earlier reports to the contrary by others.

Project Description:

PROJECT NUMBER:
Z01 HD 00131-02 NPMB

Objectives: The basic goals of the project are as follows: (1) to elucidate the basic biochemical defects responsible for selected inborn errors of metabolism in man; (2) to explore the secondary biochemical and physiological derangements caused by the primary metabolic defect; (3) to develop new or improved methods of treatment; (4) to develop new or improved methods of diagnosis, both postnatal and antenatal; and (5) to use genetically abnormal cells and patients to expand understanding of normal human biochemistry, physiology, and development. Since it is not possible for one small group of investigators to attempt to study the whole range of human biochemical defects, we have chosen to focus primarily on derangements of sulphur amino acid metabolism and secondarily on other disorders of amino acid metabolism and on selected derangements of lysosomal function. Our studies during this year have concerned the metabolic errors of glutathione synthesis and degradation (glutathionuria, glutathione synthase deficiency and gamma glutamyl-cysteine synthase deficiency), cystinosis, homocystinuria, and hyperglycinemia. Patients studied with the abnormalities of glutathione synthase include patients with severe acidosis (oxoprolinuria) and the adult, non-acidotic form of this disorder.

Methods employed: The principle methods employed are: (1) cell culture techniques, (2) spectrophotometric, radiochemical, and flourometric enzyme assays, (3) differential ultracentrifugation, (4) quantitative ion exchange chromatography of amino acids in physiological fluids, including cellular extracts, (5) radioisotope measurements of tritium, carbon-14, and sulfur-35, (6) light, phase contrast, and electron microscopy, (7) column and thin-layer chromatography, (8) starch and polyacrylamide gel electrophoresis, (9) osmometry, and (10) histochemistry.

Major Findings: Glutathionuria: In collaboration with our associated investigators, we have succeeded in identifying the two patients in the world with this particular inborn error of metabolism and have shown it to be due to a gross tissue deficiency of the enzyme gamma-glutamyl transpeptidase. This is of great interest because this enzyme has been postulated to be the mediator of mammalian aminoacid transport, and our discovery of this human mutant has permitted critical testing of this important transport hypothesis. We have demonstrated that fibroblasts deficient in gamma-glutamyl transpeptidase have normal uptake kinetics for multiple amino acids including those which are the best substrates for the transpeptidase and would presumably be most effectively transported by it in its postulated role as a transport mediator. Furthermore we have directly measured the intracellular amino acid pool sizes of the mutant fibroblasts and find them to be normal. Studies of amino acid loading and calculating renal clearances of amino acids during loading have been carried out *in vivo* with a transpeptidase deficient patient and no abnormalities in amino acid clearance even at high plasma levels have been demonstrated. These data taken together constitute strong evidence against the hypothesis that the transpeptidase is a significant mediator of mammalian amino acid transport, and these studies have begun to attract considerable attention. In the course of these studies on the transpeptidase we had performed extensive studies in normal and cystinotic tissues as detailed in last year's annual report and these succeeded in demonstrating that there is a lysosomal component of the transpeptidase activity. They also demonstrated that cystine is the most effective of the amino acid acceptors of the gamma-glutamyl group, a conclusion that has recently been supported by the published studies of other workers.

We have also studied activities for the enzymes which synthesize glutathione (as well as the transpeptidase which degrades it) in multiple human fibroblast lines and also in the liver and kidney of murine embryos and newborns. An important conclusion from these studies is that the transpeptidase appears to be a major regulator of intracellular glutathione levels during antenatal and postnatal life as well as in the tissue culture systems we have examined. Its importance in this regard has been previously overlooked by studies which focused on glutathione levels in red cells carried out by others since this enzyme is not present in red cells.

Extensive studies have been carried out on fibroblasts from patients with abnormalities in the synthesis of glutathione including the first patient discovered in the United States, by us, with oxoprolinuria. We have shown that oxoprolinuric patients have a deficient activity of glutathione synthesis in the red cells and fibroblasts and furthermore that the fibroblast enzyme demonstrates altered kinetic properties. On the basis of these and other studies, we have developed data which suggest that the red cell and fibroblast forms of glutathione synthetase are different but related enzymes. We have furthermore demonstrated a patient with the adult form of glutathione synthetase deficiency without oxoprolinuria has a different molecular lesion than most patients with oxoprolinuria, in that the adult form of the disorder is characterized by a markedly unstable form of the enzyme. The unstable enzyme appears to be responsible for the red cell deficiency in glutathione synthetase manifest by these patients; continuing synthesis of unstable enzyme in their tissues appears to protect them from marked overproduction of oxoproline like that found in patients with oxoprolinuria.

In addition we have studied patients with deficient in the first stage of glutathione synthesis (gamma-glutamyl cysteine synthase activity) and demonstrated that the two such patients known have 20-30% of normal level of enzyme presence in their cultured fibroblasts, with modest reductions in intracellular glutathione levels.

The above studies of the whole class of human inborn errors related to abnormalities of glutathione synthesis and degradation appear to us of particular importance.

As indicated in last year's report, we have undertaken several studies of cystinosis, some of which are nearing completion. We have succeeded in incorporating a number of small compounds capable of interacting with intracellular cystine into liposomes (artificial lipid vesicles) which are known *in vivo* to be selectively taken up by various tissues. Liposomes represent a mechanism for the delivery of drugs to certain stored sites in the intact organism with a minimum of toxic effects due to distribution of a test compound into such sites as the brain. We have used the fibroblast model system for studing the efficacy of liposome-enclosed compounds in depleting cystinotic intracellular cystine. Numerous compounds have been tested and the most effective has been shown to be cysteamine. The increased efficacy of liposome-enclosed cysteamine in the in vitro model system of cystinosis may prove to be of some therapeutic importance, especially as we have shown using radioactive cysteaminine that in the rat the liposome enclosed cysteaminine is distributed preferentially to the kidney, and away from brain. In the one human patient in whom non-liposome enclosed cysteaminine has been administered for the treatment of cystinosis, central nervous system toxicity was the limiting factor in treatment.

A number of clinical studies of cystinosis are also underway. One of these is a detailed study of the endocrinology of cystinosis. We have demonstrated the normalcy of adrenocortical and pituitary function in cystinosis despite growth retardation typical of the disease. However, a nearly universal abnormality in terms of diminished thyroid function-has documented. This is most easily demonstrated by an elevated level of TSH and an exaggerated response of TSH to TRF infusion. We have furthermore measured the somatomedin levels in cystinotic individuals and demonstrated that these appear normal despite a growth retardation refractory to treatment with thyroid hormone.

Another important ongoing study on cystinosis is a controlled trial of the treatment of cystinotic patients with high dose ascorbic acid, which has been demonstrated to partially deplete the intracellular cystine content of cystinotic fibroblasts. This controlled trial run collaboratively from the NIH and Dr. Jerry Schneider's laboratory in La Jolla now involves about 50 patients on whom data is being gathered. Conclusions will not be available for at least another year. In addition, a single newborn child with cystinosis is being treated in a nonrandomized manner with high dose ascorbic acid in an attempt to see if this agent will prevent expression of the earliest symptoms of the disease and we should know the answer to this question within the year.

We have now completed a study of platelet survival and the appearance of platelet ultrastructure in six patients with homocystinuria. Patients with homocystinuria have severe thromboembolic problems which are often life threatening. It has been stated that there is a reduced platelet survival in vivo in homocystinuric patients concomitant with the elevated levels of plasma homocystine. Furthermore it has been claimed that there is an abnormal platelet ultrastructure characterized by increased vaculation in homocystinuria. Detailed studies now being prepared for publication indicate that platelet survival was normal in all our homocystinuric patients and that ultrastructure was normal as well. The findings cast into doubt the proposed mechanism of thromboembolism in homocystinuria and may lead to a broader reexamination of this aspect of the disease.

Hyperglycinemia is a lethal inborn error of metabolism characterized by extremely elevated levels of plasma glycine. We have studied a patient with this disorder who has an abnormality in glycine cleavage, a reaction dependent upon reduced folic acid. Because it was considered possible that large amounts of reduced folate might stimulate glycine cleavage activities of such patients, we have undertaken the first trial of large dose leucovorin therapy in this disorder. Despite the reasonable theoretical rationale, the patient did not shown biochemical or clinical improvement on this regimen. We propose that megavitamin therapy with reduced forms of folate be attempted in other patients with this disorder, some of whom may prove to be responsive.

Significance to biomedical research and programs of the Institute. Cystinosis, homocystinuria, hyperglycinemia and the inborn errors of glutathione metabolism all present clinical pictures of varying degrees of severity, in some cases lethal. These and other heritable disorders studied in our laboratory require better methods of diagosis, treatment and prevention than are currently available and our efforts in these directions are essential for the goals of this institute. In addition it has been frequently demonstrated that the study of genetic variants in man is a powerful tool for advancing our understanding of normal and abnormal biochemistry. Our studies on glutathione metabolism illustrate that point clearly. Such understanding is an essential theme relating much significant biomedical research in diverse areas and must inevitably lead to the alleviation of medical illness characterized by genetic or nongenetic biochemical derangements.

Proposed course of project: These efforts will include: 1. The continuing study of compounds which may be of potential therapeutic benefit in cystinosis and other heritable disorders. 2. Further investigation of the mechanisms of the lysosomal metabolism of cystine and related compounds and cystinosis. 3. Continued investigation of the coagulopathy and biochemical abnormalities in homocystinuria. 4. Continued detailed studies of the enzymes responsible for the synthesis and degradation of glutathione and their involvement in disorders of glutathione metabolism. In addition to factors already studied, we intend to look at glutathione pool size and pool turnover in various mutant states, at the immunologic detection of mutant enzyme, variations of intracellular glutathione levels during normal growth and development, etc. 5. The development of an in vivo radiochemical assay for phenylalanine hydroxylase activity in patients with PKU and other abnormalities of phenylalanine metabolism. 6. Investigation of additional inborn errors of metabolism within the limitations of time and personnel, such as patients recently discovered with abnormalities of uric acid metabolism or hyperpyruvicemia.

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2. Schneider, J.A., and Schulman, J.D. The Fanconi Syndrome.In The Physiologic Basis for Disorders of Biomembranes. Andreoli, T.E., editor. Plenum Press, in press.

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6. Butler, J., Pellefigue, F., Spielberg, S.P., Schulman, J.D. Treatment of cystinotic fibroblasts with lipsome-enclosed agents. Society for Pediatric Reseach, April, 1976.

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9. Spielberg, S.P., Goodman, S.I., Tietze, F., Butler, J., Garrick, M. Kramer, L., Schulman, J.D. Glutathione metabolism in normal and mutant human cells. Society for Pediatric Research, April, 1976.

10. Mueller, P.L., Schreiber, J.R., Schulman, J.D., Ross, G.T. FSH stimulates 35S-sulfate incorporation into rat ovarian mucopolysaccharides. The Endocrine Society, June, 1976.

11. Patrick, A.D., and Schulman, J.D. Gammaglutamyl transpeptidase: Evidence of its occurrence in lysosomes of normal and cystinotic human leucocytes and normal rat liver. Biochem. Biophys. Acta. (in press).

NICHD Annual Report
July 1, 1975 through June 30, 1976

Developmental Pharmacology Branch

SUMMARY

Program Description

The newly established Developmental Pharmacology Branch studies the molecular mechanisms underlying genetic differences involved in the stimulation of pharmacologically important enzyme activities by drugs and various environmental contaminants. Why does the same dose of the same drug or other compound given to two different individuals (with the exception of monozygotic twins) evoke different responses? The responses include therapeutic effectiveness of a drug, undesirable side effects of a drug, and severe pathophysiologic processes such as cancer and birth defects. These studies are conducted with a variety of biological systems, each chosen so as to provide unique experimental access and unequivocal answers to the questions being asked. From results obtained with such experimental systems as inbred strains of mice and established cell culture lines, we can form hypotheses which can be tested in clinical patients. The Branch consists of two Sections.

The Section on Pharmacogenetics and Molecular Teratology, under the direction of Dr. Daniel W. Nebert, studies the relationship between the etiology of birth defects and the transplacental effects of these environmental stimuli on such enzyme activities. In vitro, cell culture, and inbred strains of mice are experimental systems developed for assessing and predicting teratogenic, mutagenic, and carcinogenic effects of exogenous or endogenous compounds which induce drug-metabolizing enzymes in animals and in man. The Unit on Genetic Regulation of Drug-conjugating Enzymes, under the direction of Dr. Ida S. Owens, is interested in the genetic regulation of glucuronide-conjugating enzyme induction among inbred strains of mice and in cell culture.

The Section on Molecular Toxicology, headed by Dr. Snorri S. Thorgeirsson, examines the mechanism of toxicity of carcinogenicity of N-acetylarylamines and related compounds. Repair mechanisms in mammalian cells following chemical or physical injury are studied. It is the hope of this research group to develop experimental in vitro or animal model systems for quantifying and predicting in man potentially toxic reactions of chemicals--especially drugs and environmental contaminants commonly found in the prenatal and postnatal periods.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00136-08 DPB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Pharmacogenetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	I. S. Owens	Research Biologist	DP	NICHD
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COOPERATING UNITS (if any)

SEE ATTACHED

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Developmental Pharmacology Branch

SECTION

Section on Pharmacogenetics and Molecular Teratology

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
110/12	86/12	24/12

SUMMARY OF WORK (200 words or less - underline keywords)

In certain instances numerous pharmacogenetic disorders in man cause individuals to react very differently to the same dose of the same drug. The teratogenic, carcinogenic, or toxic effects of certain drugs and other foreign compounds (xenobiotics) may also reflect important genetically mediated differences between individuals. Accordingly, our laboratory has developed experimental model systems for studying drug metabolism in cell culture and in a colony of inbred strains of mice.

We have determined that the induction of at least 10 drug-metabolizing enzyme activities is regulated at one or two genetic loci, called the Ah locus for aromatic hydrocarbon responsiveness. The relative presence of "aromatic hydrocarbon responsiveness" in certain mice results in more polycyclic hydrocarbon-initiated tumors, in a shortened sleeping time when given certain drugs, in a shortened survival time when dosed with large amounts of polycyclic hydrocarbons, in hepatic necrosis and cataract formation when given acetaminophen, and in an increased number of fetal resorptions, stillbirths, and malformations when the pregnant mother is given certain chemicals. These genetically mediated dissimilarities in xenobiotic metabolism may be a useful probe in demonstrating differences in the rates of formation of certain drug-induced birth defects.

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Project Description:

Objectives: Membrane-bound mixed-function oxygenases are important in the metabolism of drugs, carcinogens, and insecticides, as well as many endogenous lipophilic substrates such as steroids, fatty acids, cholesterol, indoles, biogenic amines, and thyroxine. We have found that the "induction," or stimulation, of a group of drug-metabolizing enzyme activities in response to aromatic hydrocarbon treatment is regulated by only a few genetic loci in the mouse. We have also employed cell culture techniques for studying the mechanism of microsomal monooxygenase induction in an experimental system which is more rigidly controlled than similar studies in the intact animal. Understanding the mechanism by which drug-metabolizing enzymes are induced may elucidate important concepts in the fields of drug metabolism, developmental pharmacology, steroid biochemistry, chemical carcinogenesis, teratology, entomology, and limnology. The main objectives of this project are (1) to exploit genetic differences in drug metabolism between various inbred strains of mice in order to understand better the potential importance of pharmacogenetics; (2) to use tissue culture techniques in answering questions which would be difficult or impossible with studies in the intact animal; (3) to combine the genetic differences with the tissue culture techniques in order to elucidate certain mechanisms related to mammalian gene regulation; and (4) to apply certain of these techniques to human tissues so that extrapolations can be made from rigidly controlled experimental systems to pharmacogenetic differences in man.

Methods Employed: The principal methods employed are: (1) recording spectrophotometry of turbid solutions, (2) enzyme assays involving radiometric, spectrophotometric, and spectrophotofluorometric determinations of product formation or substrate disappearance, (3) radioisotopic measurements using tritium and carbon-14, (4) cell culture techniques, (5) differential centrifugation, (6) phase microscopy, (7) equilibrium dialysis, (8) polyacrylamide gel electrophoresis, (9) column- and thin-layer chromatography, (10) magnetic resonance techniques, (11) high pressure liquid chromatography, and (12) gas chromatography.

Major Findings: (1) The kinetics of aryl hydrocarbon (benzo[a]pyrene hydroxylase induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)--among 10 established cell lines, fetal primary cultures derived from hamster, rat, chick, rabbit, and 4 inbred strains of mice, and cultured human lymphocytes--is very similar to the kinetics of hydroxylase induction by 3-methylcholanthrene (MC) as the inducer. (2) The TCDD-inducible process is sensitive to actinomycin D and cycloheximide at levels of inhibitor similar to those previously reported with MC in culture. (3) The induced enzyme activity in cells treated with TCDD plus MC is not greater than that in cells exposed to either inducer alone. (4) There exists no relationship between cytotoxicity by TCDD and the level of inducible hydroxylase activity in culture. (5) Estimated ED_{50} values for the hydroxylase induction by TCDD range from about 0.12 nM in C57BL/6N mouse cultures and 0.23 nM in the H-4-II-E cell line to more than 100 nM in the VERO and HTC cell lines. No hydroxylase activity is detectable in the control, MC-, or TCDD-treated LB82 cell line. (6) In several cell lines and in primary cultures, the responsiveness of hydroxylase induction to TCDD is between 250 and 900 times greater than that to MC.

(7) The responsiveness of hydroxylase induction to TCDD in C57BL/6N mouse-derived cultures is about 16 times greater than the responsiveness to TCDD in DBA/2N mouse-derived cultures; this difference in responsiveness to TCDD is very similar to that seen in these two mouse strains in vivo. (8) A bioassay with H-4-II-E cells is suggested for the detection of minute (10^{-14} mole) levels of TCDD. (9) A bacterial mutagenesis assay and genetic differences in microsomal CO-binding cytochromes were combined in vitro to evaluate the metabolic activation of several known carcinogens to frameshift mutagens. With the use of liver fractions from C57BL/6N and DBA/2N control mice and mice treated in vivo with 3-methylcholanthrene, β-naphthoflavone, phenobarbital, or 2,3,7,8-tetrachlorodibenzo-p-dioxin, the in vitro mutagenicity of 3-methylcholanthrene, 6-aminochrysene, and 2-acetylaminofluorene--but not benzo[a]pyrene--is closely associated with the genetically mediated difference in both aromatic hydrocarbon-inducible aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity and new cytochrome P_1450 formation. (10) 3-Methylcholanthrene, 6-aminochrysene, and 2-acetylaminofluorene--but not benzo[a]pyrene--are each more mutagenic in vitro per molecule of cytochrome P_1450 than per molecule of CO-binding cytochrome other than P_1450. (11) Diethylmaleate, and especially 1,1,1-trichloro-2,3-epoxide, increases the mutagenicity of benzo[a]pyrene, whereas no increases occur with 3-methylcholanthrene, 6-aminochrysene, or 2-acetylaminofluorene activation to mutagens in vitro. (12) Differences in toxicity caused by various environmental pollutants or drugs were studied in several inbred strains of mice and in siblings of the (C57BL/6N)(DBA/2N)F_1 X DBA/2N backcross, in which the phenotypes aromatic hydrocarbon "responsiveness" or "nonresponsiveness" had been predetermined. "Responsiveness" is associated with shortened survival times following large (500 mg kg^{-1} day $^{-1}$) doses of intraperitoneal benzo[a]pyrene, 7,12-dimethylbenz[a]anthracene, 3-methylcholanthrene, β-naphthoflavone, or polychlorinated biphenyls; the cause of death in these experiments is not yet certain. "Nonresponsiveness" is associated with shortened survival times following small (1 to 2 mg kg^{-1} day $^{-1}$) oral doses of benzo[a]pyrene, 7,12-dimethylbenz[a]anthracene, 3-methylcholanthrene, or lindane, yet is associated with a longer survival time following small daily doses of oral polychlorinated biphenyls. (13) All nonresponsive mice ingesting benzo[a]pyrene daily die within 4 weeks, whereas the survival of responsive mice ingesting benzo[a]pyrene daily is not significantly different from that of control mice; the apparent cause of early death in these experiments is toxic depression of the bone marrow. (14) When given an intraperitoneal dose of lindane that is lethal to normal mice within 12 hours, 3-methylcholanthrene-pretreated responsive mice are protected and therefore do not die during this time period. (15) A dose of bromobenzene sufficient to cause considerable necrosis in the liver of a pregnant mouse does not cause transplacentally any detectable necrosis in fetal liver. (16) These data suggest (i) that the life span of animals exposed to certain environmental compounds can be markedly influenced by a single gene or a very small number of genes; and (ii) that the same genetic trait can be either beneficial or detrimental to the animal, depending on whether detoxification or metabolic potentiation occurs. (17) Cordycepin, ethidium bromide, and puromycin aminonucleoside treatment of the mouse tumor line Hepa-1 produce strikingly different effects on the kinetics of hydroxylase induction, when the inducers benz[a]anthracene, phenobarbital, and isoproterenol are compared. (18) Without any of these "usual inducers" present

in the mouse hepatoma cell line, 0.50-5.0 mM puromycin aminonucleoside or 40 nM actinomycin-D cause large increases in the hydroxylase activity--at a time when gross RNA synthesis is virtually 100% inhibited and gross protein synthesis is more than 50% inhibited. (19) This aminonucleoside- or actinomycin-D-mediated induction process in the Hepa-1 cell line represents the first demonstration of aryl hydrocarbon hydroxylase "induction" without prior treatment with usual inducers and hence presumably without prior accumulation of putative induction-specific RNA; moreover, this induction process apparently requires little, or no simultaneous RNA synthesis during exposure to the aminonucleoside or actinomycin-D. In contrast, when primary cultures derived from normal rat liver are treated with similar high concentrations of the aminonucleoside, no induction of the hydroxylase activity occurs. This result suggests that the effect of puromycin aminonucleoside on hydroxylase-specific mRNA synthesis, processing, or stabilization is very different between the hepatoma cell line and normal fetal rat primary hepatocytes in culture. (20) Eight inducible monooxygenase activities, previously shown to be associated with the genetic locus (or loci) controlling the formation of mouse cytochrome P_1-450, were studied in liver and kidney microsomes from rabbit. Despite spectral evidence for cytochrome P_1-450 induction in rabbit liver, of these eight activities only the N- and ring-hydroxylation reactions involving two N-acetylarylamines, 2-acetylaminofluorene and acetanilide, are induced more than 2-fold. Four of the monooxygenase activities not inducible in rabbit liver are, however, inducible in rabbit kidney. These data support the hypothesis that there are multiple forms of inducible cytochrome P_1-450 that differ from organ to organ as well as from species to species. (21) Aryl hydrocarbon hydroxylase induction in the eye from inbred C57BL/6N (responsive) and DBA/2N (nonresponsive) mice and from offspring of the appropriate genetic crosses is apparently under the same genetic regulation as that in liver. The hydroxylase activity is 2- to more than 10-fold inducible in the eye by intraperitoneally administered polycyclic hydrocarbons such as β-naphthoflavone, 3-methylcholanthrene, and 2,3,7,8-tetrachlorodibenzo-p-dioxin. (22) Histofluorescent examination of the eye from β-naphthoflavone-treated C57BL/6N mice indicates that the hydroxylase activity is closely associated with the retinal pigmented epithelium-choroid layer; the retina demonstrates little activity. It is proposed that genetically controlled induction of the hydroxylase activity and perhaps other cytochrome P-450-mediated monooxygenase activities in the retinal pigmented epithelium-choroid play an important role for detoxification and/or metabolic potentiation of drugs and other xenobiotics. (23) The hydroxylase activity can be induced in cultured cells of chick embryonic retinal pigmented epithelium by 1,2-benz[a]anthracene. (24) All known mouse strains which develop retinal degeneration are aromatic hydrocarbon responsive strains. Some of the major products of the aryl hydrocarbon hydroxylase reaction, phenolic benzo[a]pyrene derivatives, have a marked labilizing effect on lysosomal membranes. On the basis of these two observations, a possible involvement of the hydroxylase activity in initiating retinal degeneration is suggested. (25) Electrophoresis of liver microsomal proteins from inbred C57BL/6N and DBA/2N and recombinant inbred AKXL-38 and AKXL-38A mouse strains revealed the presence of four polypeptides whose relative staining intensity could be correlated with the induction state of the microsomes as determined by enzymatic and spectral methods. Of these four bands, band 4 (55,000 daltons) was increased whenever spectral measurements revealed an increase in the cytochrome P-448 content due to administration of β-naphthoflavone or 2,3,7,8-tetrachlorodibenzo-p-dioxin. (26) Administration of pregnenolone-16α-carbonitrile caused an increase in band 3 (54,000 daltons), whereas administration of phenobarbital caused an increase primarily in band 2 (51,000 daltons) but also smaller increases in band 1 (49,000 daltons) and band 4. The changes observed for phenobarbital and pregnenolone-16α-carbonitrile were the same for both responsive and nonresponsive strains. (27) Electrophoretic experiments combined with double-labeling of radioisotopes indicate that the increased formation of cytochrome P-448 and P-450 by β-naphthoflavone and phenobarbital, respectively, is primarily the result of an increased rate of de novo protein synthesis rather than a decreased degradation rate or a conversion of pre-existing polypeptides. (28) Benzo[a]pyrene was incubated in vitro with cofactors and liver microsomes from rats previously treated with β-naphthoflavone, and the polycyclic hydrocarbon and its metabolites were extracted with a benzene-acetone mixture and examined by room-temperature electron spin resonance (ESR) spectroscopy. Contrary to reports from other laboratories, the ESR signal representing an oxybenzo[a]pyrene free radical is initially very small or undetectable. (29) Incubations with benzo[a]pyrene, cofactors, and microsomes under an atmosphere of $^{17}O_2$ demonstrate that the source of oxygen in the radical is atmospheric and the reaction is mediated by cytochrome P-448 or P-450 but that the free radical we observe occurs nonenzymically-presumably via abstraction of a hydrogen atom (one-electron air oxidation) by molecular oxygen in the solvent used for ESR analysis. (30) The various forms of cytochrome P-450 in mouse liver or skin microsomes produce reactive metabolites of BP that bind to DNA in vitro. These results confirm the work of other laboratories in which rat liver microsomes or cell cultures were used. Nine peaks, indicating BP metabolites bound to DNA nucleosides, can be reproducibly identified from C3H, C57BL/6, and DBA/2 mice. (31) When either the C3H or C57BL/6 strain is compared with the DBA/2 strain, a positive general correlation is found between the carcinogenic index in vivo and each of three determinations in vitro: the size of eight of the nine peaks, the hepatic aryl hydrocarbon hydroxylase activity, and the total hepatic cytochrome P-450 content. Peaks D, E, H, and I demonstrate relative differences among the three strains that are in the same direction as the carcinogenic index in vivo, i.e. the peaks are greater in C3H than in C57BL/6 and both are considerably greater than those in DBA/2 mice. (32) When C3H mice are compared with C57BL/6 mice, however, no quantitative correlation is found between the carcinogenic index and the size or shape of any of the nine peaks, the hepatic hydroxylase activity, or the total hepatic cytochrome P-450 content. Hence, the total quantity of nucleoside-BP metabolites in each of the nine peaks is not necessarily associated with biological activity (i.e. cancer in vivo). (33) A defect in the general immune competence of the C3H inbred strain may be an important factor in its markedly increased susceptibility to BP-initiated tumorigenesis, compared with the C57BL/6 strain. (34) With hepatic microsomes, peak G is associated, at least in part, with nucleoside-metabolite complexes involving BP-4,5-oxide, whereas the other eight peaks are associated predominantly with metabolites formed by cytochrome P_1-450 rather than other forms of P-450. (35) With skin microsomes, all peaks including peak G appear to be associated with metabolites formed predominantly by cytochrome P_1-450. Peak E is associated predominantly with BP-7,8-diol-9,10-epoxide bound to one or more nucleosides. This reactive intermediate, oxygenated in the non-K region, is associated with genetically mediated increases in aromatic hydrocarbon-inducible cytochrome P_1-450. (36) The induction of four hepatic monooxygenase activities--naphthalene trans-1,2-dihydrodiol formation, acetanilide 4-hydroxylase, biphenyl 4-hydroxylase, and biphenyl 2-hydroxylase--by polycyclic aromatic compounds is genetically associated with the induction of aryl hydrocarbon hydroxylase activity and cytochrome P_1-450 in C57BL/6N and DBA/2N inbred mice and among progeny from the appropriate genetic crosses involving these two progenitor strains. (37) These enzyme activities were studied with respect to (i) preferential inhibition by metyrapone, α-naphthoflavone, or Tween 80 in vitro; (ii) use of the microsomal inducers 3-methylcholanthrene, β-naphthoflavone, 2,3,7,8-tetrachlorodibenzo-p-dioxin, or phenobarbital; (iii) apparent K_m values; and (iv) heat inactivation. (38) Several lines of evidence suggest that aromatic hydrocarbon-inducible naphthalene trans-1,2-dihydrodiol formation, acetanilide 4-hydroxylase, and biphenyl 4-hydroxylase activities are, like the induced aryl hydrocarbon hydroxylase activity, associated with cytochrome(s) P_1-450 and that these three control enzyme activities are, like control aryl hydrocarbon hydroxylase activity, associated with one (or more) forms of P-450 other than cytochrome(s) P_1-450. The basal and aromatic hydrocarbon-inducible biphenyl 2-hydroxylase activity in mouse liver, on the other hand, appears to be associated solely with cytochrome(s) P_1-450. (39) A pair of closely related sublines that differ with respect to the Ah locus (for aromatic hydrocarbon responsiveness) were used to identify or confirm the pleiotropic effects of this gene. The lines were derived by sibling-mating without selection from (C57L/J x AKR/J)F_2 mice; the two sublines were separated at the F_{12} generation. Ten microsomal monooxygenase activities and one cytosol enzyme activity known to be associated with the Ah locus were similarly associated with cytochrome P_1-450 formation in these recombinant inbred sublines as well. Nine additional hepatic monooxygenase activities studied were found not to be associated with the Ah locus. (40) The Ah^b-containing subline was highly susceptible to 3-methylcholanthrene-induced subcutaneous sarcomas, whereas the Ah^d-containing subline was relatively resistant. (41) Genetic differences in aryl hydrocarbon hydroxylase induction in cultured mitogen-activated human lymphocytes by 3-methylcholanthrene appear to be controlled by one genetic locus, and relatively high inducibility may be associated with increased susceptibility to bronchogenic carcinoma. With 16 pairs of normal adult twins, we attempted to determine whether the hydroxylase inducibility in cultured lymphocytes is a reliable indicator of genetic differences in the plasma clearance of several drugs. Although fold inducibility remained reasonably constant for an individual, a wide variation in absolute hydroxylase activity was noted from one determination to another. (42) Certain findings indicated that variable (unknown) constituents in different lots of fetal calf serum might induce the hydroxylase activity by mechanisms different from induction by 3-methylcholanthrene, thereby making fold inducibility an unreliable indicator of genotype. Nonetheless, the index of heritability (h^2) for fold inducibility in twins was fairly high (0.67). (43) There was no correlation between the hydroxylase activity in culture and the biologic half-life of antipyrine, phenylbutazone, or dicoumarol in these twins. (44) The allele for responsiveness (Ah^b) is shown to be associated with (i) increases in the apparent K_s values for metyrapone bound to reduced P-450; (ii) increases in the ethylisocyanide difference ratio ($\Delta A_{455-490}/\Delta A_{430-490}$); (iii) increases in the $\Delta A_{455-490}$ per mg of microsomal protein but not in the $\Delta A_{430-490}$ per mg of protein from the reduced P-450-ethylisocyanide complex; (iv) an approximately 2-nm hypsochromic shift in the spectral maximum in the 446-nm region for the reduced P-450-metyrapone complex; (v) an approximately 2-nm hypsochromic shift of the absorption maximum in the 455-nm region, but not of the maximum in the 430-nm region, for the reduced P-450-ethylisocyanide complex; and (vi) larger increases in the $\Delta A_{455-490}$ than in the $\Delta A_{430-490}$ per mg of microsomal protein for the reduced P-450-ethylisocyanide complex as a function of increasing pH. All of these phenomena are felt to be associated with the genetically regulated induction of liver microsomal cytochrome P-448 by polycyclic aromatic compounds. (45) Whereas increases in the total hepatic P-450 content appear to be expressed almost exclusively as a single autosomal dominant trait, the increase in apparent K_s value for metyrapone bound to reduced P-450 appears to be expressed additively. (46) Although total P-450 content does not increase in C57BL/6N mice treated with 3-methylcholanthrene plus cycloheximide, hepatic P-448 induction does occur; P-448 induction does not occur in DBA/2N mice under these same conditions. These results indicate that cytochrome P-448 induction is relatively resistant to the inhibition of protein synthesis and that a responsive animal treated with 3-methylcholanthrene plus cycloheximide cannot be considered experimentally the same as a genetically nonresponsive animal treated with 3-methylcholanthrene alone. (47) Using a radiometric assay for specific binding of [^{3}H]TCDD to cytosol protein, we found a soluble saturable high affinity binding protein which may act as a receptor in the initial step of AHH induction by TCDD. The apparent K_d for TCDD (0.5-1.0 nM) in TCDD-"responsive" Hepa-1 and Reuber H-4 lines compared favorably with the ED_{50} for AHH induction (0.23-0.45 nM). The apparent K_d in TCDD-"nonresponsive" HTC and VERO lines (1.5-2.0 nM), on the other hand, was much less than the ED_{50} for AHH induction (100->200 nM), indicating that the inability of these lines to respond to induction by TCDD reflects some defect other than an absence of soluble binding sites. (48) Radiolabelled TCDD was displaced from these sites by nonlabelled TCDD but not by high concentrations of analogs which are ineffective in inducing AHH activity. (49) Hepatic microsomes were examined on the day of preparation with both a low-temperature (either 77° or 9.5° K) electron paramagnetic resonance (EPR) spectrometer and a room-temperature spectrophotometer. Our findings indicate that (i) the hydrophobic character or availability of lone-pair electrons at various positions on the molecule of the test compound and (ii) spin state of the P-450 iron--at the time the test compound interacts with P-450--are the two most important determinants in producing a Type I, Type II, Reverse Type I, or no difference spectrum at all and the magnitude of the peak-to-trough absorbance of the spectrum. (50) Using the responsive C57BL/6 and the nonresponsive DBA/2 inbred strains, the progeny of the F_1 X DBA/2 backcross, and 8 other responsive or nonresponsive inbred strains, and using β-naphthoflavone, TCDD, or phenobarbital as inducers, we found that ornithine decarboxylase activity increases 8 to 18 hours after inducer treatment and that this increase is directly correlated with the $\underline{Ah}^b$ allele. These data strongly suggest that, although the number of genes regulated by the Ah locus is miniscule compared with the total population of genes, one can detect gene activation (via ornithine decarboxylase induction) during treatment with polycyclic aromatic compound inducers.

Significance to Biomedical Research and the Program of the Institute: The cytochrome P-450-mediated monooxygenase systems metabolize steroids, cholesterol, fatty acids, indoles, sympathomimetic amines, and thyroxine, as well as lipophilic foreign substrates. Administration of polycyclic aromatic hydrocarbons to the pregnant animal produces induction of this enzyme activity in the placenta and also transplacental stimulation of the hydroxylase in several fetal tissues. Cigarette smoking is associated with increases in human placental aryl hydrocarbon hydroxylase activity. Ingestion of charcoal-broiled beef by rats is associated with increased intestinal metabolism of several drugs. We now have demonstrated that what appears to be a single-gene difference--"aromatic hydrocarbon responsiveness"--in the mouse is associated with: (1) increased susceptibility to 3-methylcholanthrene-initiated tumors; (2) decreased zoxazolamine paralysis time; (3) increased susceptibility to 7,12-dimethylbenz[a]anthracene-produced skin inflammation; (4) increased susceptibility to acetaminophen-induced hepatic necrosis and cataract formation; (5) shortened survival time, or (conversely) apparent protection, when large doses of polycyclic hydrocarbons, lindane, or polychlorinated biphenyls are administered intraperitoneally or orally; (6) apparent protection against aplastic anemia caused by oral benzo[a]pyrene; and (7) increased susceptibility to stillborns, fetal resorptions, and malformations caused by 3-methylcholanthrene or 7,12-dimethylbenz[a]anthracene given to the pregnant mother. An increased incidence of lung cancer in man has been correlated with living in highly polluted urban areas. It is therefore possible that differences--caused by a single gene or a very small number of genes--in man are related to increased susceptibility to cancers, tissue necrosis, drug metabolism, bone marrow disorders, and decreased life span caused by exposure to foreign compounds.

These genetically mediated dissimilarities in xenobiotic metabolism may be a useful probe in demonstrating differences in the rates of formation of certain drug-produced birth defects. Consequently, studies involving the development and extent of induction of mixed-function oxygenases in fetal tissues and in the placenta during various periods of gestation, are relevant to the program of this Institute in the following areas: (1) possible effects of environmental pollutants (i.e., polycyclic hydrocarbons and insecticides) and various drugs on normal fetal growth and development and on the health of the pregnant woman, (2) mechanisms of "pharmacological immaturity" of the premature and newborn, (3) mechanisms of disease of the fetus and newborn concerning oxygenative and conjugative metabolism of normal body substrates such as steroids and bilirubin, and (4) possible therapeutic and diagnostic procedures for aiding and predicting pharmacogenetic defects in the fetus and neonate.

Proposed Course of Project: (1) Delineate further differences between monooxygenase induction by phenobarbital and that by polycyclic hydrocarbons, (2) characterize specific and nonspecific binding of these various inducers to subcellular sites, (3) examine the effects of hormones and nutrition on monooxygenase induction and associated intermediary molecules of importance such as cyclic-AMP, cyclic-GMP, and polyamines, (4) study various inhibitors and activators of monooxygenase activity in cell culture, (5) determine differences in metabolite formation, binding of reactive metabolic intermediates to DNA, and biophysical characteristics of the enzyme active-site between control and drug-treated animals or cells in culture, (6) compare these results in genetic differences with contemporary models of mammalian gene expression and regulation, and (7) search for drug metabolism parameters in cultured human lymphocytes or fibroblasts or in various excreta that might aid in predicting a patient's response to a drug or class of drugs.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00137-02 DP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Genetic Regulation of Drug-Conjugating Enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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LAB/BRANCH
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SECTION
Pharmacogenetics and Molecular Teratology

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
15/12	12/12	3/12

SUMMARY OF WORK (200 words or less - underline keywords) Detoxification of certain highly lipophilic endogenous substrates and noxious foreign compounds, such as environmental pollutants, requires a multistep process involving phase I and phase II enzyme systems. Previously, with inbred strains of mice, research in this Branch has established that certain monooxygenases (phase I enzymes) are regulated at a few genetic loci and that expression of enzyme induction is dominant or codominant in response to aromatic hydrocarbon treatment. Recently, studies were initiated to understand the impact of secondary reactions on the detoxification process, since the products of the monooxygenase system can serve as substrate for conjugation via the UDP glucuronyl transferase system (phase II enzymes). In hepatic tissue from aromatic hydrocarbon treated mice, it has beenshown that induction of a transferase enzyme system lags behind the time course of induction of a certain monooxygenase system, is genetically linked to the induction of the monooxygenase, and is expressed as a codominant trait in progeny from backcrosses and intercrosses of the prototype strains of mice. Since many noxious foreign compounds are metabolized initially via the monooxygenase system and subsequently by the transferase system or one of several other drug-metabolizing enzymes, an understanding of the functional relationship of these various enzymes might explain why carcinogenesis, toxicity, teratogenesis, and/or mutagenesis are caused by certain xenobiotics and not by others.

Project Description:

Objectives: Certain membrane-bound mixed-function monooxygenase activities in various tissues of certain inbred strains of responsive mice rise after treatment with some aromatic hydrocarbons. On the contrary, these enzyme activities in tissue from inbred strains of nonresponsive mice do not rise following aromatic hydrocarbon treatment. Determinations of a number of different monooxygenase activities in progeny from crosses and backcrosses of responsive and nonresponsive mice show that several inducible monooxygenase activities always increase concomitantly, that expression of enzyme induction can be dominant or codominant, and that induction is regulated by a small number of genetic loci. Monooxygenases are important in the metabolism of drugs, carcinogens, and insecticides, as well as many endogenous lipophilic substrates such as thyroxine, steroids, fatty acids, and cholesterol. With the necessary cofactors these exogenous and endogenous lipophilic compounds are transformed to oxygenated labile intermediates or stable products *via* this enzyme system. This initial step in drug detoxification is designated a "phase I reaction." Other membrane-bound drug-metabolizing enzymes act to modify intermediates or stable products formed by phase I reactions, thereby completing the transformation of lipophilic compounds to highly water-soluble entities for efficient urinary excretion. These secondary steps in drug metabolism are designated "phase II reactions." For example, epoxide hydratase may transform highly reactive epoxide intermediates formed by the monooxygenase into less reactive dihydrodiols; epoxide-glutathione transferase(s) convert react epoxides to innocuous glutathione conjugates, and UDP glucuronyl transferase(s) conjugates various hydroxylated products to the glucuronic acid moiety of UDP glucuronic acid. These conjugates are the most water-soluble derivatives of the original compound. Therefore, both drug hydroxylation and subsequent glucuronide conjugation are necessary for the most efficient removal of certain noxious lipophilic compounds from the body. An important question is: to what extent are these two processes coupled in drug metabolism? In the mouse genetic model system of responsiveness and nonresponsiveness for monooxygenase activities after aromatic hydrocarbon treatment, the induction of hepatic membrane-bound UDP glucuronyl transferase activity, with at least one substrate, lags behind the time course of induction of the monooxygenase activities, is genetically linked to the induction of the monooxygenase, and is expressed as a codominant trait in progeny from backcrosses and intercrosses of the prototype inbred strains. These observations suggest that hydroxylation of highly lipophilic compounds by monooxygenases is required to generate hydroxylated substrates to be conjugated to the glucuronic acid moiety of UDP glucuronic acid *via* UDP glucuronyl transferase. Thus, an understanding of the relationship between regulation and function of phase I and phase II drug-metabolizing enzymes may elucidate and explain observations related to developmental pharmacology, drug metabolism, chemical carcinogenesis, and teratology that have heretofore remained obscure. The primary objectives of this project are: (1) to explore further the genetically mediated induction of UDP glucuronyl transferase, using other substrates with known specific monooxygenase requirements, in an attempt to determine whether there exists distinct classes of substrates for the induced transferase; (2) to explore further whether the parent polycyclic hydrocarbon or its oxygenated intermediate is the proximal inducer of the transferase; (3) to determine whether induced UDP glucuronyl transferase, as assayed *in vitro*, reflects the increased in vivo conjugation of certain endogenous and exogenous substrates; (4) to exploit genetic differences in the conjugation of critical substrates, as bilirubin; (5) to study the functional interrelationship between UDP glucuronyl transferase and various P450-mediated monooxygenases in the detoxification of various drugs in vivo in genetically different mouse strains; (6) to use the bacterial mutagenesis system developed by Ames to examine the effect of UDP glucuronyl transferase on the mutagenicity of certain compounds. (It is well known that inhibition of β-glucuronidase by specific inhibitors reduces bladder tumors in animal models. Classically, β-glucuronidase reverses the action of UDP glucuronyl transferase); (7) to attempt to use tissue culture techniques to study the regulation of mammalian UDP glucuronyl transferase activity and the interdependence in regulation of the transferase and the monooxygenase at the molecular level; and (8) to apply tissue culture results for the purposes of establishing a bioassay for "breast milk jaundice factor."

Methods Employed: The principal methods to be employed are: (1) enzyme assays using spectrophotometric, spectrophotofluorometric, radiometric determinations of product or disappearance of substrate, (2) radiometric measurements using tritium and carbon-14, (3) cell culture techniques, (4) differential centrifugation, (5) phase microscopy, (6) high pressure liquid chromatography, and (7) column chromatography, and (8) variable temperature regulators.

Major Findings: (1) After aromatic hydrocarbon treatment and with 4-methyl-7-hydroxyumbelliferone as the substrate, hepatic UDP glucuronyl transferase activity increases in five inbred strains of mice, whereas transferase activity in five other inbred strains does not increase. (2) Aromatic hydrocarbon treatment of F_1 hybrids from crosses between C57BL/6N (B6) and DBA/2N (D2) causes an increase in transferase activity intermediate in levels between that of the two inbred parents. (3) In one-half of the offspring from a cross between (B6D2)F_1 hybrids and the nonresponsive D2 parent and after aromatic hydrocarbon treatment, hepatic transferase activity is induced to intermediate levels between that of the inbred parents, and in the other one-half of the offspring activity is not induced. (4) Likewise, in one-half of the offspring from a cross between (B6D2)F_1 hybrids and the responsive B6 parent after such treatment, transferase activity is induced to intermediate levels, and in the other one-half of the offspring activity is fully induced to the same levels as the responsive inbred parent. (5) In F_2 progeny from a cross of two (B6D2)F_1 hybrids, after hydrocarbon treatment, hepatic transferase activity was induced fully in approximately one-fourth of the offspring, was induced to intermediate levels in one-half of the offspring, and was not induced to any extent in approximately one-fourth of the offspring. These data from the offspring of these two different types of backcrosses and the one intercross indicate that transferase induction is gene-dose dependent, with heterozygotes one-half as responsive as the responsive inbred parent. Determinations of both hepatic microsomal aryl hydrocarbon hydroxylase (a monooxygenase) and UDP glucuronyl transferase activities in each offspring show that the genes which control aromatic hydrocarbon induction of monooxygenase cosegregate with the gene(s) which control aromatic hydrocarbon induction of UDP glucuronyl transferase activity, yielding the same pattern of inheritance in the offspring of the various genetic crosses and backcrosses. Monooxygenase activity, however, is essentially dominantly expressed in the heterozygote.

(6) Transferase activity is maximal not until 3 to 5 days after 3-methylcholanthrene (3-MC) or β-naphthoflavone (BNF) treatment, whereas aryl hydrocarbon hydroxylase activity is essentially maximal 24 hours after treatment. 3-Methylcholanthrene is approximately twice as effective an inducer of transferase as BNF. (7) 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a potent inducer of aryl hydrocarbon hydroxylase in responsive and nonresponsive mice, induces the transferase activity about 4-fold in responsive C57BL/6N mice and about 2-fold in the nonresponsive DBA/2N. The rise in activity in the C57BL/6N mice proceeds without a lag, reaches a maximum in 12 days and slowly declines to basal levels after about 100 days. Induction in the DBA/2N mice starts after a 3 day lag, reaches a maximum in 8 days and rapidly returns to basal levels by 20 days. The dramatic difference in the effects of TCDD on the transferase activity in the two strains of mice is strong additional evidence for genetically mediated induction of transferase activity. (8) Administration of TCDD to generate equally induced levels of aryl hydrocarbon hydroxylase in the B6 and D2 mice, followed by administration of 3-methylcholanthrene, does not induce further the hepatic transferase activity in either strain. Prior hydroxylase induction by the very potent and nonmetabolized inducer, TCDD, to equal levels of enzyme activity in both responsive and nonresponsive mice presumably neutralizes any genetic differences between these two strains with respect to their capacity to generate, in vivo, hydroxylated products of 3-methylcholanthrene. The failure of the D2 mouse to respond to elevated levels of hydroxylated 3-methylcholanthrene suggests: (a) the product of the monooxygenase reaction is not the proximal inducer for transferase and/or, (b) that possibly a second defect, different from the putative defective aromatic hydrocarbon-inducer receptor, exists as a barrier to transferase induction by aromatic hydrocarbons in the nonresponsive mouse. (9) In the responsive B6 parent after 3-methylcholanthrene treatment, hepatic transferase activity is not induced before parturition, is slightly induced for the first three weeks postnatally, and is maximally induced at four to eight weeks of age. Activity in the nonresponsive D2 parent is not induced at any developmental stage. The latency of native microsomal UDP glucuronyl transferase activity, requiring detergent treatment for maximal expression in vitro, is not age-dependent with respect to activation by triton X-100. (10) Heat inactivation studies with hepatic microsomes from control or methylcholanthrene-treated B6 and D2 mice indicate that methylcholanthrene treatment does not alter heat lability. Transferase from D2 mice, however, appears to be more sensitive to heat than that of the B6 mice. (11) With the hepatoma cell-line H-4-II-E in cell culture, a transferase activity with the substrate 4-methyl-7-hydroxyumbelliferone is induced at least 2-fold by the aromatic hydrocarbon, benz[a]anthracene. This is the first demonstration to my knowledge of an inducible transferase system in cell culture.

Significance to Biomedical Research and the Program of the Institute: As discussed previously, drug oxygenations and subsequent glucuronide conjugations are catalyzed by a membrane-bound multistep enzyme complex. These enzymes are important in the process of detoxification and elimination of many normal body constituents and many xenobiotics taken in from our environment in the form of food additives, air pollutants, and medication. There are, as well, diseases of humans ascribed to defects (not completely understood) in drug-conjugating enzymes. Examples in pediatrics include neonatal hyperbilirubinemia, Gilbert's syndrome, and the Crigler-Najjar syndrome. Therefore, the development of experimental model systems for understanding better the molecular regulation and neonatal development of UDP glucuronyl transferase is pertinent to the program of the institute in the following areas: (1) understanding the mechanisms of "immaturity of drug metabolism" in neonates; (2) providing further experimental models for other human pharmacogenetic defects; (3) elucidating the basic enzymology of glucuronidating drug metabolism in order to gain insight into the development of selective inhibitors and stimulators of these drug conjugating enzymes that are useful for beneficial regulation of abnormal metabolism peculiar to various human diseases; (4) understanding metabolism of various drugs in the fetus, premature and the newborn.

Proposed Course of Project: (1) Further exploration of the genetic difference in response to aromatic hydrocarbon treatment between certain inbred strains of mice to determine substrate preferences for the induced transferase; (2) examination of urine and bile for glucuronides of labelled exogenous substrates administered to treated responsive and nonresponsive mice; (3) determination of time course for glucuronide and other metabolites of the monooxygenase system, with a comparison of various carcinogenic and noncarcinogenic polycyclic hydrocarbons in the two genetic strains of mice; (4) further characterization of the transferase system in cell culture with respect to macromolecular syntheses requirements, potential inhibitors, and activators and general regulation at the molecular level; (5) comparative studies of transferase inducibility in cell culture by nonmetabolized inducers versus their parent oxygenated metabolites; (6) determination of bilirubin tolerance in responsive and nonresponsive mouse after hydrocarbon treatment; (7) examination of the effect of UDP glucuronyl transferase on bacterial mutagenesis; (8) development of a useful bioassay in cell culture for the quantitation of "breast milk jaundice factor" in human breast milk samples from outpatients and/or inpatients; and (9) purification of UDP glucuronyl transferase, with the intention of developing an antibody to the enzyme.

Publications: NONE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00138-02 DP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mechanism of Toxicity and Carcinogenicity of N-acetylarylamines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
64/12	52/12	12/12

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of this project is to study the mechanism of toxicity and carcinogenicity of N-acetylarylamines and related compounds. The topics of present interest are: 1) The relationship between the induction of aryl hydrocarbon hydroxylase and N-hydroxylation of N-acetylarylamines after administration of polycyclic aromatic hydrocarbons to the inbred strains of mice C57BL/6N and DBA/2N and their progeny as well as other species. 2) The role of glutathione in protecting the developing fetus and the adult animals from the harmful effects of electrophilic metabolites arising from N-acetylarylamines. 3) The mechanism of species differences in N-hydroxylation of N-acetylarylamines in liver microsomes. 4) The relationship between mutagenicity and carcinogenicity of N-acetylarylamines. 5) The effect of partial hepatectomy on metabolism and carcinogenicity of N-acetylarylamines in mice. 6) The role of vitamin A in modifying the covalent binding of N-acetylarylamines to hepatic DNA. 7) The characterization of monooxygenase(s) system in ovaries from rats and mice and its potential role in chemically induced ovarian cancer.

Project Description:

Objectives: The hepatotoxicity and carcinogenicity of N-acetylarylamines such as acetaminophen and 2-acetylaminofluorene have been shown to result from toxic metabolites arising via N-oxidation. Resently N-oxidation of these compounds has been proven to be mediated by the cytochrome P450-dependent monooxigenase system(s). Inducers of cytochrome P450-associated monooxygenase activity, such as 3-methylcholanthrene and phenobarbital, are known to modify both the toxicity and carcinogenicity of N-acetylarylamines. It has been shown previously that administration of polycyclic hydrocarbons such as 3-methylcholanthrene increases the activity of another monooxygenase, aryl hydrocarbon (benzo[a]pyrene) hydroxylase in the livers of certain inbred strains of mice but not other strains. Furthermore, it has been demonstrated that the induction of aryl hydrocarbon hydroxylase activity by 3-methylcholanthrene segregates as a single autosomal dominant gene in progeny from the appropriate backcrosses and intercrosses involving C57BL/6 (responsive strain) and DBA/2 (nonresponsive strain) mice. We have shown that the genetically mediated presence or absence of induction, as well as the magnitude of induction, of aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity is highly correlated with the N-hydroxylation of 2-acetylaminofluorene in the livers of C57BL/6N and DBA/2N inbred mice treated with the microsomal enzyme inducers 3-methylcholanthrene, β-naphthoflavone, 2,3,7,8-tetrachlorodibenzo-p-dioxin, and sodium phenobarbital. The extent of hepatotoxicity caused by acetaminophen (p-hydroxyacetanilide) administered intraperitoneally to these two strains of mice is also highly associated with both aromatic hydrocarbon-inducible monooxygenase "activities": aryl hydrocarbon hydroxylase and acetylarylamine N-hydroxylase. We have suggested that cytochrome P_1450 is associated with the aromatic hydrocarbon-inducible N-hydroxylase activity and that these genetic differences among inbred strains of mice offer a valuable experimental model system for studying the mechanism of hepatotoxicity and carcinogenicity among siblings of a defined genotype. Vitamin A has recently been shown to have a "protecting effect" against induction of cancer by chemical carcinogens both in tissue culture and in the whole animal. The mechanism by which vitamin A exerts this protective affect is not yet known, but two modes of action appear most probable. Many of the chemical carcinogens which vitamin A protects against need to be metabolized into reactive alkylating agents before they can initiate their carcinogenic effect. The enzyme system responsible for this activation is the cytochrome P450-dependent monooxygenases. Hence, one possibility for the mechanism of protection is that vitamin A inhibits directly the monooxygenase system. A second possibility is that vitamin A induces the DNA repair enzymes and thereby increases the rate of turnover of DNA which has been damaged by reactive alkylating agents or metabolites. The urban population is exposed to increasing amounts of environmental pollutants in the form of polycyclic hydrocarbons, insecticides, food additives, etc. Many of these pollutants, such as DDT and the polycyclic hydrocarbons, are potent inducers of the cytochrome P450-dependent monooxygenase(s) system, which metabolizes a variety of drugs and foreign compounds. Metabolism of these drugs and foreign compounds results, in most cases, in more polar and therefore more easily excreted metabolites. In some cases, however, the relatively non-reactive parent drugs and foreign compounds, are activated through the cytochrome P450 system to reactive electrophiles, which may cause a variety of toxic effects, including cell death, cancer, fetal malformations, and blood disorders. The main objectives of this project are: (1) to study the relationship between the induction of aryl hydrocarbon hydroxylase and N-hydroxylation of N-acetylarylamines after administration of the polycyclic hydrocarbon 3-methylcholanthrene to the inbred strains of mice and other species; (2) to examine the role of N-hydroxylation of N-acetylarylamines in various tissues from different animal species, including man, in causing mutagenicity and/or carcinogenicity; (3) to clarify and define the mechanism(s) by which vitamin A protects against chemically induced cancer in the "responsive" and "nonresponsive" inbred strains of mice; (4) to examine the role of glutathione in protecting the developing fetus and the adult animal from the harmful effects of electrophilic metabolites produced either by the mother or by its own enzyme systems; and (5) to examine the effect of partial hepatectomy on polycyclic hydrocarbon induction of N-hydroxylation on N-acetylarylamines and incidence of liver tumors after administration of N-acetylarylamines.

Methods Employed: The principal methods employed are: (1) recording spectrophotometry of turbid solutions, (2) enzyme assays involving radiometric, spectrophotometric, and spectrophotofluorometric determinations of product formation or substrate disappearance, (3) radioisotopic measurements using tritium and carbon-14, (4) tissue culture techniques, (5) differential centrifugation, (6) phase microscopy, (7) equilibrium dialysis, (8) polyacrylamide gel electrophoresis, (9) column- and thin-layer chromatography, (10) gas chromatography, and (11) high pressure liquid chromatography.

Major Findings: (1) We have extended our studies in inbred strains of mice on the genetic control of polycyclic hydrocarbon induced N-hydroxylation of N-acetylarylamines to include two commonly used drugs acetaminophen (Tylenol$^{(R)}$) and phenacetin. N-hydroxylation of both acetaminophen and phenacetin, yielding metabolites capable of causing liver necrosis and methemoglobinemia, shows a strong genetic association with aryl hydrocarbon (benzo[a]pyrene) hydroxylase (AHH) activity in the polycyclic hydrocarbon responsive and nonresponsive mice. Furthermore, the degree of toxicity, such as liver necrosis, is highly correlated with AHH activity. Implication of this finding in man is being examined. (2) 3-Methylcholanthrene (MC) treatment induces 2-acetylaminofluorene N-hydroxylase (ANH), acetanilide hydroxylase (AH), aryl hydrocarbon hydroxylase (AHH), and at least 7 other monooxygenase activities and hepatic cytochrome P_1450 in genetically "responsive" C57BL/6 (B6) but not in "nonresponsive" DBA/2 (D2) inbred mice. The response in MC-treated rats is very similar to that in responsive mice. MC treatment of rabbits stimulates P_1450 formation and ANH and AH but not AHH, p-nitroanisole O-demethylase, 7-ethoxycoumarin O-deethylase, biphenyl-2- or 4-hydroxylase, or naphthalene hydroxylase activity. MC-treated hamsters have induced ANH and AHH activities and cytochrome P_1450, whereas MC-treated guinea pigs have little, if any, increase in ANH or AHH or P_1450 content. α-Naphthoflavone (ANF) in vitro is known to inhibit preferentially the induced ANH and AHH activities in MC-treated B6 mice or rats but not these enzymes in MC-treated D2 mice and not the basal ANH or AHH activity in mice or rats. ANF selectively inhibits the basal and MC-induced ANH activities but not AHH activity in control or MC-treated rabbits. These data indicate that there exist differing substrate specificities for cytochrome P_1450 among these five animal species. (3) The genetically mediated difference in aromatic hydrocarbon-inducible hepatic aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity is associated with in vitro metabolic activation of 2-acetylaminofluorene to a mutagen by liver fractions. With the use of "responsive" C57BL/6N and "nonresponsive" DBA/2N inbred strains and offspring from the appropriate crosses, the aromatic hydrocarbon-inducible hydroxylase activity appears to be expressed as an autosomal dominant trait, whereas 2-acetylaminofluorene mutagenicity in vitro appears to be expressed additively. With N-hydroxy-2-acetylaminofluorene added in vitro, no metabolic activation is necessary for mutagenesis to occur; however, mutagenicity is enhanced 20- to 40-fold in the presence of liver fractions. The metabolic activation of N-hydroxy-2-acetylaminofluorene to a frameshift mutagen in vitro is not associated with the genetically mediated difference in aromatic hydrocarbon responsiveness. We therefore suggest that the rate-limiting step of 2-acetylaminofluorene mutagenesis is its activation by cytochrome $P_1$450 to the N-hydroxy derivative, which is metabolized further to a much more mutagenic intermediate by a reaction independent of cytochrome $P_1$450--possibly a deacetylation reaction. (4) 2-Acetylaminofluorene (2-acetyl-AF), a known carcinogen, is also a potent mutagen. Both the carcinogenicity and mutagenicity of 2-acetyl-AF depend upon metabolism, primarily N-hydroxylation and subsequent esterification or deacetylation. The structural features necessary for mutagenic activity, were examined in more than 25 analogs of 2-acetyl-AF in the Ames assay, with and without 9000 x g supernatant from mouse liver. Analogs prepared included 2-propionyl, -butyryl, -t-butylacetyl, -benzoyl-AF; heteroatom-substituted 2-acetyl derivatives, e.g., bromoacetyl, mono-, di- and trichloroacetyl, methoxyacetyl-AF; various olefinic analogs including acrylyl and HC=C-CO-AF. Carbamate derivatives [2-COOR, R=Me,Ph] and urea derivatives [2-CO-NRR', R,R'=H,Me,Ph] were also prepared. All compounds were mutagenic in the presence, but none in absence of 9000 x g supernatant. The mutagenic potency of these analogs showed that steric features appear to be more important than electronic characteristics. Straight chain homologs of 2-acetyl-AF are nearly equally mutagenic as 2-acetyl-AF; however, when bulk of the carbon skeleton about the acyl group is increased, the mutagenicity is decreased. Carbamate and urea analogs are also potent mutagens indicating all are substrates for N-hydroxylation and any subsequent steps necessary for mutagenesis. (5) The effect of partial hepatectomy (67-75% excised) on aryl hydrocarbon (benzo[a]pyrene) hydroxylase (AHH) and 2-acetylaminofluorene N-hydroxylase activities in responsive C57BL/6N (B6) and nonresponsive DBA/2N (D2) inbred strains of mice were studied. The basal AHH activity in B6 mice is reduced 70% 12 hours after the operation, but returns to control values between 36 and 48 hours and remains at that level thereafter. Similar, but less pronounced, changes are seen in the D2 mouse 3-methylcholanthrene (MC) treatment (80 mg kg^{-1} i.p.) 1 hour after partial hepatectomy induces AHH in B6 mice 48 hours later to the same extent as in B6 mice with intact livers. In the partially hepatectomized D2 mice, MC has no effect on AHH activity 48 hours later. Similar effect after partial hepatectomy and MC treatment is observed for 2-acetylaminofluorene N-hydroxylase in both B6 and D2 mice. In view of the known involvement of certain monooxygenases such as AHH and the N-hydroxylase in metabolically potentiating certain foreign compounds, this experimental model offers a valuable approach to further studies of the role of these cytochrome P-450-mediated enzymes in causing toxicity and cancer. (6) Studies have been initiated to examine the mechanism of metabolic activation of AAF and the role of AHH in the initiation of hepatic tumors after administration of AAF or BP to B6 or D2 mice and offspring from the appropriate genetic backcrosses and intercross. A specific monooxygenase cytochrome P_1450 (or P-448) is known to catalyze the N-hydroxylation of AAF, and this reaction is competitively inhibited by AAF analogs such as N-methoxycarbonyl-2-aminofluorene, N-propionyl-2-aminofluorene, and N-(n-butyryl)-2-aminofluorene. These and other AAF analogs are metabolically activated by the cytochrome P_1450 system to potent mutagens, as determined by the Salmonella histidine revertant in vitro system developed by Ames. In order to increase the susceptibility of the liver to AAF- or BP-initiated tumors, we performed partial hepatectomies before administration of these compounds. Preliminary results indicate a strong association between genetically controlled P_1-450-mediated metabolism of AAF or BP induced by PH and the occurrence of tumors. (7) Large doses of acetaminophen (Tylenol$^{(R)}$ cause fulminant hepatic necrosis in both man and experimental animals. Hepatic necrosis caused by acetaminophen parallels the formation of a toxic metabolite(s) that binds covalently to liver proteins and glutathione is very important in protecting the liver against the toxic metabolite(s). We have examined various developmental aspects of acetaminophen metabolism in mice. The glutathione content in the liver reached adult levels at 10 days of age. The rate of cytochrome P_1450-dependent metabolism of acetaminophen, as measured by percent glutathione depletion, was slow until day 10, then rapidly reached adult levels by day 15. An increase in covalently bound acetaminophen metabolite(s) was found to be coincident with the development of cytochrome P_1450-dependent metabolic pathways. Our results suggest that the glutathione-dependent detoxification pathway matures prior to the maturation of the drug-metabolizing enzymes which convert acetaminophen into the toxic metabolite(s) in mice. (8) Existing epidemiologic data suggest that exposure during the first 20 years of life to environmental factors, such as chemical pollutants, may be important in the etiology of human ovarian cancer. Most of these pollutants are not carcinogenic themselves but require metabolic activation by microsomal cytochrome P-450-dependent monooxygenases. To explore the relationship between the exposure of chemicals to the ovary and the metabolism of chemicals by the ovary, we studied the effect of 3-methylcholanthrene (MC) treatment on ovarian aryl hydrocarbon (benzo[a]pyrene) hydroxylase (AHH) and N-acetylarylamine N-hydroxylase activities. Three strains of sexually mature female rats (Sprague-Dawley, Lewis, and Brown Norway [BN/SsN]) were treated with MC (80 mg/kg intraperitoneally) 40 hours prior to sacrifice. AHH activity was induced in the ovary from all three strains: highest (about 5-fold) in Sprague-Dawley and lowest (2.5-fold) in BN/SsN. There was a good correlation, as a function of time, among (i) induced AHH activity in ovary, (ii) induced N-hydroxylase activity in ovary, and (iii) increased mutagenesis in vitro when either 2-acetylaminofluorene or N-hydroxy-2-acetylaminofluorene is combined with ovarian tissue homogenate (500 x g supernatant fraction) and assayed with the Salmonella histidine revertant experimental system developed by Ames. (9) Vitamin A has been shown to have "protecting effect" against induction of cancer by chemical carcinogens both in tissue culture and in the whole animal. We have found that vitamin A, either in physiological concentration or concentrations several times that, does not inhibit the cytochrome P_1-450-dependent metabolism of benzo[a]pyrene or 2-acetylaminofluorene in the liver or the lung of inbred strains of mice. We are currently examining the effect of vitamin A and other retinoids on DNA repair enzymes both in bacteria and mammalian cells.

Significance to Biomedical Research and the Program of the Institute: Acetaminophen is a widely used drug by people of all ages. The 1975 Physicians' Desk Reference contains 60 preparations containing acetaminophen. Of particular interest is the fact that acetaminophen is the most widely used mild analgesic in pediatric medicine. Chemically, acetaminophen belongs to a class of compounds called N-acetylarylamines. The N-acetylarylamine class also includes compounds such as N-acetylaminofluorene, which has been widely used as a model compound for induction of chemical carcinogenesis. Both the drug acetaminophen and the carcinogen N-acetylaminofluorene are metabolized by the same enzyme system in the liver into reactive arylating metabolites. This enzyme system (i.e. cytochrome P450-dependent monooxygenases) can be modified by drugs such as the barbiturates and environmental pollutants such as polycyclic hydrocarbons. Consequently, studies involving examination of the mechanism of activation of the N-acetylarylamines into toxic compounds, the reaction of the body to these toxic compounds, and the possible protection of these reactive alkylating compounds are relevant to the program of this Institute in the following areas: (1) Possible effects of environmental pollutants (i.e. polycyclic hydrocarbons and insecticides) and various drugs on normal fetal growth and development--in particular, the incidence of malformation in the newborn exposed in utero to drugs known to be metabolized into reactive alkylating metabolite(s). (2) Mechanism of pharmacological and toxicological responses in the premature and newborn. (3) Possible preventive and/or therapeutic procedures for aiding and/or predicting pharmacogenetic defects in the fetus and neonate.

As a result of our studies, identification of those who have high aryl hydrocarbon hydroxylase levels--which has been correlated with bronchogenic carcinoma in man--might now be possible with the use of a single small dose of the drug acetaminophen and the examination of urinary metabolites.

Proposed Course of Project: (1) Further delineation of the relationship between aryl hydrocarbon hydroxylase activity and N-hydroxylation of N-acetylarylamines in the inbred strains of mice following pretreatment with polycyclic hydrocarbons; (2) detailed examination of the catalytic enzyme-site responsible for N-hydroxylation of N-acetylarylamines; (3) developmental studies of N-hydroxylation and C-hydroxylation of N-acetylarylamines in the inbred C57BL/6 and DBA/2 strains, and their relationship to toxicity and carcinogenicity of these compounds; (4) exploration of the possibility of "phenotyping" for aryl hydrocarbon hydroxylase and N-hydroxylase activity in man by measuring the amount of mercapturic acid conjugate following oral administration of acetaminophen; (5) further studies on mutagenicity of N-acetylarylamines with emphasis on cytosol enzymes such as transacetylase, sulfotransferase and deacetylase; (6) role of the liver microsomal mixed function amine oxidase (non-cytochrome P-450-dependent) in toxicity and mutagenicity; (7) further studies on the effect of partial hepatectomies in mice on cytochrome P-450-dependent metabolism and carcinogenicity of N-acetylarylamines and polycyclic hydrocarbons; (8) further delination of the role of ovarian monooxygenase in chemically induced ovarian cancer;

(9) modification of carcinogenicity of N-acetylarylamines by steroids; (10) effects of vitamin A on DNA repair enzymes in both bacterial and mammalian cells.

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7. Thorgeirsson, S.S., and Nelson, W.L.: Separation and quantitative estimation of 2-acetylaminofluorene and its hydroxylated metabolites by high pressure liquid chromatography. Anal. Biochem., in press.

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